PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/52, 19/00, 16/24, A61K 38/19

A2

(11) International Publication Number: **WO 97/05250**

(43) International Publication Date: 13 February 1997 (13.02.97)

(21) International Application Number: PCT/FI96/00427

(22) International Filing Date: 1 August 1996 (01.08.96)

(30) Priority Data:

08/510,133	1 August 1995 (01.08.95)	US
08/585,895	12 January 1996 (12.01.96)	US
08/601,132	14 February 1996 (14.02.96)	US
08/671,573	28 June 1996 (28.06.96)	US

(71) Applicant *(for all designated States except US)*: HELSINKI UNIVERSITY LICENSING LTD. OY [FI/FI]; Viikinkaari 8 A, FIN-00710 Helsinki (FI).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ALITALO, Kari [FI/FI]; Nyyrikintie 4 A, FIN-02100 Espoo (FI). JOUKOV, Vladimir [RU/FI]; Topeliuksenkatu 32 G 8, FIN-00290 Helsinki (FI).

(74) Agent: OY JALO ANT-WUORINEN AB; Iso Roobertinkatu 4-6 A, FIN-00120 Helsinki (FI).

(81) Designated States: AU, CA, CN, JP, NO, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.
With an indication in relation to a deposited microorganism furnished under Rule 13bis separately from the description.
Date of receipt by the International Bureau:
21 August 1996 (21.08.96)

(54) Title: RECEPTOR LIGAND VEGF-C

(57) Abstract

Provided are polypeptide ligands for the receptor tyrosine kinase, Flt4. Also provided are cDNAs and vectors encoding the ligands, pharmaceutical compositions and diagnostic reagents comprising the ligands, and methods of making ans using the ligands.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

RECEPTOR LIGAND VEGF-C

This application is a continuation-in-part of United States Patent Application Serial No. 08/671,573, filed June 28, 1996, which is a continuation-in-part of United States Patent Application Serial Number 08/601,132, filed February 14, 1996, which is a continuation-in-part of United States Patent Application Serial Number 08/585,895, filed January 12, 1996, which is a continuation-in-part of United States Patent Application Serial Number 08/510,133, filed August 1, 1995. This application also is a continuation-in-part of United States Patent Application Serial Number 08/340,011, filed November 14, 1994.

FIELD OF THE INVENTION

The present invention generally relates to the field of genetic engineering and more particularly to growth factors for endothelial cells and growth factor genes.

BACKGROUND OF THE INVENTION

Developmental growth, the remodelling and regeneration of adult tissues, as well as solid tumor growth, can only occur when accompanied by blood vessel formation. Angioblasts and hematopoietic precursor cells differentiate from the mesoderm and form the blood islands of the yolk sac and the primary vascular system of the embryo. The development of blood vessels from these early (*in situ*) differentiating endothelial cells is termed vasculogenesis. Major embryonic blood vessels are believed to arise via vasculogenesis, whereas the formation of the rest of the vascular tree is thought to occur as a result of vascular sprouting from pre-existing vessels, a process called angiogenesis, Risau *et al.*, *Devel. Biol.*, *125*:441-450 (1988).

Endothelial cells give rise to several types of functionally and morphologically distinct vessels. When

organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases. Upon angiogenic stimulation, endothelial cells may re-enter the cell cycle, migrate, withdraw from the cell cycle and subsequently differentiate again to form new vessels that are functionally adapted to their tissue environment. Endothelial cells undergoing angiogenesis degrade the underlying basement membrane and migrate, forming capillary sprouts that project into the perivascular stroma. Ausprunk *et al.*, *Microvasc. Rev.*, *14*:51-65 (1977). Angiogenesis during tissue development and regeneration depends on the tightly controlled processes of endothelial cell proliferation, migration, differentiation, and survival. Dysfunction of the endothelial cell regulatory system is a key feature of many diseases. Most significantly, tumor growth and metastasis have been shown to be angiogenesis dependent. Folkman *et al.*, *J. Biol. Chem.*, *267*:10931-10934 (1992).

Key signals regulating cell growth and differentiation are mediated by polypeptide growth factors and their transmembrane receptors, many of which are tyrosine kinases. Autophosphorylated peptides within the tyrosine kinase insert and carboxyl-terminal sequences of activated receptors are commonly recognized by kinase substrates involved in signal transduction for the readjustment of gene expression in responding cells. Several families of receptor tyrosine kinases have been characterized. Van der Geer *et al.*, *Ann. Rev. Cell Biol.*, *10*:251-337 (1994). The major growth factors and receptors transducing angiogenic stimuli are schematically shown in Fig. 1.

Fibroblast growth factors are also known to be involved in the regulation of angiogenesis. They have been shown to be mitogenic and chemotactic for cultured endothelial cells. Fibroblast growth factors also stimulate the production of proteases, such as

collagenases and plasminogen activators, and induce tube formation by endothelial cells. Saksela *et al., Ann. Rev. Cell Biol., 4*:93-126 (1988). There are two general classes of fibroblast growth factors, FGF-1 and FGF-2, both of which lack conventional signal peptides. Both types have an affinity for heparin and FGF-2 is bound to heparin sulfate proteoglycans in the subendothelial extracellular matrix from which it may be released after injury. Heparin potentiates the stimulation of endothelial cell proliferation by angiogenic FGFs, both by protecting against denaturation and degradation and dimerizing the FGFs. Cultured endothelial cells express the FGF-1 receptor but no significant levels of other high-affinity fibroblast growth factor receptors.

Among other ligands for receptor tyrosine kinases, the platelet derived growth factor, PDGF-BB, has been shown to be weakly angiogenic in the chick chorioallantoic membrane. Risau *et al., Growth Factors,* 7:261-266 (1992). Transforming growth factor α (TGFα) is an angiogenic factor secreted by several tumor cell types and by macrophages. Hepatocyte growth factor (HGF), the ligand of the c-*met* proto-oncogene-encoded receptor, also is strongly angiogenic.

Recent evidence shows that there are endothelial cell specific growth factors and receptors that may be primarily responsible for the stimulation of endothelial cell growth, differentiation and certain differentiated functions. The best studied of these is vascular endothelial growth factor (VEGF), a member of the PDGF family. Vascular endothelial growth factor is a dimeric glycoprotein of disulfide-linked 23 kD subunits. Other reported effects of VEGF include the mobilization of intracellular calcium, the induction of plasminogen activator and plasminogen activator inhibitor-1 synthesis, stimulation of hexose transport in endothelial cells, and promotion of monocyte migration *in vitro*. Four VEGF isoforms, encoded by distinct mRNA splice

variants, appear to be equally capable of stimulating mitogenesis in endothelial cells. However, each isoform has a different affinity for cell surface proteoglycans, which behave as low affinity receptors for VEGF. The 121 and 165 amino acid isoforms of VEGF (VEGF121 and VEGF165) are secreted in a soluble form, whereas the isoforms of 189 and 206 amino acid residues remain cell surface-associated and have a strong affinity for heparin. VEGF was originally purified from several sources on the basis of its mitogenic activity toward endothelial cells, and also by its ability to induce microvascular permeability, hence it is also called vascular permeability factor (VPF).

The pattern of VEGF expression suggests its involvement in the development and maintenance of the normal vascular system and in tumor angiogenesis. During murine development, the entire 7.5 day post-coital (p.c.) endoderm expresses VEGF and the ventricular neuroectoderm produces VEGF at the capillary ingrowth stage. *See* Breier *et al., Development, 114*:521-523 (1992). On day two of quail development, the vascularized area of the yolk sac as well as the whole embryo show expression of VEGF. In addition, epithelial cells next to fenestrated endothelia in adult mice show persistent VEGF expression, suggesting a role in the maintenance of this specific endothelial phenotype and function.

Two high affinity receptors for VEGF have been characterized. These are VEGFR-1/Flt-1 (fms-like tyrosine kinase-1) and VEGFR-2/Kdr/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1). Those receptors are classified in the PDGF-receptor family, but they have seven rather than five immunoglobulin-like loops in their extracellular domain (see Fig. 1) and they possess a longer kinase insert than normally observed in this family. The expression of VEGF receptors occurs mainly in vascular endothelial cells, although some may be present on hematopoietic progenitor cells, monocytes,

and melanoma cells. Only endothelial cells have been reported to proliferate in response to VEGF, and endothelial cells from different sources show different responses. Thus, the signals mediated through VEGFR-1 and VEGFR-2 appear to be cell type specific. The VEGF-related placenta growth factor (PlGF) was recently shown to bind to VEGFR-1 with high affinity. PlGF was able to enhance the growth factor activity of VEGF, but it did not stimulate endothelial cells on its own. Naturally occurring VEGF/PlGF heterodimers were nearly as potent mitogens as VEGF homodimers for endothelial cells. Cao *et al.*, *J. Biol. Chem.*, *271*:3154-62 (1996).

The Flt4 receptor tyrosine kinase (VEGFR-3) is closely related in structure to the products of the VEGFR-1 and VEGFR-2 genes. Despite this similarity, the mature form of Flt4 differs from the VEGF receptors in that it is proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides. Pajusola *et al.*, *Cancer Res.*, *52*:5738-5743 (1992). The 4.5 and 5.8 kb Flt4 mRNAs encode polypeptides which differ in their C-termini due to the use of alternative 3′ exons. Isoforms of VEGF or PlGF do not show specific binding to Flt4 or induce its autophosphorylation.

Expression of Flt4 appears to be more restricted than the expression of VEGFR-1 or VEGFR-2. The expression of Flt4 first becomes detectable by *in situ* hybridization in the angioblasts of head mesenchyme, the cardinal vein, and extraembryonically in the allantois of 8.5 day p.c. mouse embryos. In 12.5 day p.c. embryos, the Flt4 signal is observed in developing venous and presumptive lymphatic endothelia, but arterial endothelia appear negative. During later stages of development, Flt4 mRNA becomes restricted to developing lymphatic vessels. The lymphatic endothelia and some high endothelial venules express Flt4 mRNA in adult human tissues and increased expression occurs in lymphatic sinuses in metastatic lymph nodes and in lymphangioma.

These results support the theory of the venous origin of lymphatic vessels.

Five endothelial cell specific receptor tyrosine kinases, Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), Flt4 (VEGFR-3), Tie, and Tek/Tie-2 have so far been described, which possess the intrinsic tyrosine kinase activity essential for signal transduction. Targeted mutations inactivating Flt-1, Flk-1, Tie, and Tek in mouse embryos have indicated their essential and specific roles in vasculogenesis and angiogenesis at the molecular level. VEGFR-1 and VEGFR-2 bind VEGF with high affinity (K_d 16 pM and 760 pM, respectively) and VEGFR-1 also binds the related placenta growth factor (PlGF; K_d about 200 pM). A ligand for Tek is reported in PCT patent publication WO 96/11269.

SUMMARY OF THE INVENTION

The present invention provides a ligand, designated VEGF-C, for the Flt4 receptor tyrosine kinase (VEGFR-3). Thus, the invention provides a purified and isolated polypeptide which is capable of binding to the Flt4 receptor tyrosine kinase. Preferably, an Flt4 ligand of the invention is capable of stimulating tyrosine phosphorylation of Flt4 receptor tyrosine kinase in a host cell expressing the Flt4 receptor tyrosine kinase. Preferred ligands of the invention are mammalian polypeptides. Highly preferred ligands are human polypeptides. As explained in detail below, dimers and multimers comprising polypeptides of the invention linked to each other or to other polypeptides are specifically contemplated as ligands of the invention.

In one embodiment, an FLT4 ligand polypeptide has a molecular weight of approximately 23 kD as determined by SDS-PAGE under reducing conditions. For example, the invention includes a ligand composed of one or more polypeptides of approximately 23 kD which is purifyable from conditioned media from a PC-3 prostatic

adenocarcinoma cell line, the cell line having ATCC Acc. No. CRL 1435. Amino acid sequencing of this PC-3 cell derived ligand polypeptide revealed that the ligand polypeptide comprises an amino terminal amino acid sequence set forth in SEQ ID NO: 13. A conditioned medium comprising an Flt4 ligand is itself an aspect of the invention. The present invention also provides a new use for the PC-3 prostatic adenocarcinoma cell line which produces an Flt4 ligand. In a preferred embodiment, the ligand may be purified and isolated directly from the PC-3 cell culture medium.

In a highly preferred embodiment, the ligand polypeptide comprises a fragment of the amino acid sequence shown in SEQ ID NO: 33 which specifically binds to the human Flt4 receptor tyrosine kinase. Exemplary fragments include: a polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 33 from about residue 112 to about residue 213; a polypeptide comprising an amino acid sequence from about residue 104 to about residue 227 of SEQ ID NO: 33; and a polypeptide comprising an amino acid sequence from about residue 112 to about residue 227 of SEQ ID NO: 33. Other exemplary fragments include polypeptides comprising amino acid sequences of SEQ ID NO: 33 that span, approximately, the following residues: 31-213, 31-227, 32-227, 103-217, 103-225, 104-213, 113-213, 103-227, 113-227, 131-211, 161-211, 103-225, 227-419, 228-419, 31-419, and 1-419, as described in greater detail below.

The present invention also provides one or more polypeptide precursors of an Flt4 ligand, wherein one such precursor (designated "prepro-VEGF-C") comprises the complete amino acid sequence (amino acid residues 1 to 419) shown in SEQ ID NO: 33. Thus, the invention includes a purified and isolated polypeptide having the amino acid sequence of residues 1 to 419 shown in SEQ ID NO: 33. Ligand precursors according to the invention, when expressed in an appropriate host cell, produce, via

cleavage, a polypeptide which binds specifically to the Flt4 receptor tyrosine kinase. A putative 102 amino acid leader (prepro) peptide has been identified in the amino acid sequence shown in SEQ ID NO: 33. Thus, in a related aspect, the invention includes a purified and isolated polypeptide having the amino acids sequence of residues 103-419 shown in SEQ ID NO: 33.

In one embodiment, an expressed Flt4 ligand polypeptide precursor is proteolytically cleaved upon expression to produce an approximately 23 kD Flt4 ligand polypeptide. Thus, an Flt4 ligand polypeptide is provided which is the cleavage product of the precursor polypeptide shown in SEQ ID NO: 33 and which has a molecular weight of approximately 23 kD under reducing conditions.

A putative VEGF-C precursor or splice variant, consisting of polypeptides with molecular weights of about 29 and 32 kD, also is considered an aspect of the invention.

In another embodiment, an expressed Flt4 ligand polypeptide precursor is proteolytically cleaved upon expression to produce an approximately 21 kD VEGF-C polypeptide. Sequence analysis has indicated that an observed 21 kD form has an amino terminus approximately 9 amino acids downstream from the amino terminus of the 23 kD form, suggesting that alternative cleavage sites exist.

From the foregoing, it will be apparent that an aspect of the invention includes a fragment of the purified and isolated polypeptide having the amino acid sequence of residues 1 to 419 shown in SEQ ID NO: 33, the fragment being capable of specifically binding to Flt4 receptor tyrosine kinase. Preferred embodiments include fragments having an apparent molecular weight of approximately 21/23 kD and 29/32 kD as assessed by SDS-PAGE under reducing conditions.

Evidence suggests that the amino acids

essential for retaining Flt4 ligand activity are contained within approximately amino acids 103/112-226/227 of SEQ ID NO: 33, and that a carboxy-terminal proteolytic cleavage to produce a mature, naturally-occurring Flt4 ligand occurs at the approximate position of amino acids 226-227 of SEQ ID NO: 33. Accordingly, a preferred Flt4 ligand comprises approximately amino acids 103-227 of SEQ ID NO: 33.

VEGF-C mutational analysis described herein indicates that a naturally occurring VEGF-C polypeptide spanning amino acids 103-227 of SEQ ID NO: 33, produced by a natural processing cleavage that defines the C-terminus, exists and is biologically active as an Flt4 ligand. A polypeptide fragment consisting of residues 104-213 of SEQ ID NO: 33 has been shown to retain VEGF-C biological activity. Additional mutational analyses indicate that a polypeptide spanning only amino acids 113-213 of SEQ ID NO: 33 retains Flt4 ligand activity. Accordingly, preferred polypeptides comprise sequences spanning, approximately, amino acid residues 103-227, 104-213, or 113-213, of SEQ ID NO: 33.

Moreover, sequence comparisons of members of the VEGF family of polypeptides provide an indication that still smaller fragments will retain biological activity, and such smaller fragments are intended as aspects of the invention. In particular, eight highly conserved cysteine residues of the VEGF family of polypeptides define a region from residue 131 to residue 211 of SEQ ID NO: 33 (see Figures 10 & 31); therefore, a polypeptide spanning from about residue 131 to about residue 211 is expected to retain VEGF-C biological activity. In fact, a polypeptide comprising approximately residues 161-211, which retains an evolutionarily-conserved RCXXCC motif, is postulated to retain VEGF-C activity, and therefore is intended as an aspect of the invention. Some of the conserved cysteine residues in VEGF-C participate in interchain disulfide

bonding to make homo- and heterodimers of the various naturally occurring VEGF-C polypeptides. Beyond the preceding considerations, evidence exists that VEGF-C polypeptides lacking interchain disulfide bonds retain VEGF-C biological activity. Consequently, the materials and methods of the invention include all VEGF-C fragments that retain at least one biological activity of VEGF-C, regardless of the presence or absence of interchain disulfide bonds. The invention also includes multimers (including dimers) comprising such fragments linked to each other or to other polypeptides. Fragment linkage may be by way of covalent bonding (*e.g.*, disulfide bonding) or non-covalent bonding of polypeptide chains (*e.g*, hydrogen bonding, bonding due to stable or induced dipole-dipole interactions, bonding due to hydrophobic or hydrophilic interactions, combinations of these bonding mechanisms, and the like).

In yet another related aspect, the invention includes variants and analogs of the aforementioned polypeptides, including VEGF-C, precursors of VEGF-C, and fragments of VEGF-C. The variants contemplated by the invention include purified and isolated polypeptides having amino acid sequences that differ from the amino acid sequences of VEGF-C, VEGF-C precursors and VEGF-C fragments by conservative substitutions, as recognized by those of skill in the art, or by additions or deletions of amino acid residues that are compatible with the retention of at least one biological activity of VEGF-C.

Analogs contemplated by the invention include polypeptides having modifications to one or more amino acid residues that differ from the modifications found in VEGF-C, VEGF-C precursors, or VEGF-C fragments, but are compatible with the retention of at least one biological activity of VEGF-C, VEGF-C precursors, or VEGF-C fragments. For example, analogs within the scope of the invention include glycosylation variants and conjugants (attachment of the aforementioned polypeptides to

compounds such as labels, toxins, etc.)

The present invention also provides purified and isolated polynucleotides (*i.e.*, nucleic acids) encoding polypeptides of the invention, for example a cDNA or corresponding genomic DNA encoding a VEGF-C precursor, VEGF-C, or biologically active fragments or variants thereof. A preferred nucleic acid of the invention comprises a DNA encoding amino acid residues 1 to 419 of SEQ ID NO: 33 or one of the aforementioned fragments thereof. Due to the degeneracy of the genetic code, numerous such coding sequences are possible, each having in common the coding of the amino acid sequence shown in SEQ ID NO: 33 or fragment thereof. The invention also comprehends analogs of these polynucleotides, or derivatives of any one of these polynucleotides that encodes a polypeptide which retains at least one biological activity of VEGF-C. DNA polynucleotides according to the invention include genomic DNAs, cDNAs, and oligonucleotides comprising the coding sequence for a fragment of VEGF-C, or an analog of a VEGF-C fragment that retains at least one of the biological activities of VEGF-C. Distinct polynucleotides encoding a polypeptide of the invention by virtue of the degeneracy of the genetic code are within the scope of the invention. In one embodiment, the invention contemplates polynucleotides having sequences that differ from polynucleotides encoding a VEGF-C fragment in a manner that results in conservative amino acid differences between the encoded polypeptides, as understood by those of skill in the art.

A preferred polynucleotide according to the invention comprises the human VEGF-C cDNA sequence set forth in SEQ ID NO: 32 from nucleotide 352 to 1611. Other polynucleotides according to the invention encode a VEGF-C polypeptide from, *e.g.*, mammals other than humans, birds (e.g., avian quails), and others. Still other polynucleotides of the invention comprise a coding

sequence for a VEGF-C fragment, and allelic variants of those DNAs encoding part or all of VEGF-C.

The invention further comprises polynucleotides that hybridize to the aforementioned polynucleotides under standard stringent hybridization conditions. Exemplary stringent hybridization conditions are as follows: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM $Na \cdot PO_4$, pH 6.8 and washing in 0.2X SSC at 55°C. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining appropriate hybridization conditions. *See* Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Second ed., Cold Spring Harbor Laboratory Press 1989) §§ 9.47-9.51. These polynucleotides, capable of hybridizing to polynucleotides encoding VEGF-C, VEGF-C fragments, or VEGF-C analogs, are useful as nucleic acid probes for identifying, purifying and isolating polynucleotides encoding other (non-human) mammalian forms of VEGF-C. Additionally, these polynucleotides are useful in screening methods of the invention, as described below.

Preferred nucleic acid probes of the invention comprise nucleic acid sequences of at least about 16 continuous nucleotides of SEQ ID NO: 32. More preferably, these nucleic acid probes would have at least about 20 nucleotides found in a subsequence of SEQ ID NO: 32. In using these nucleic acids as probes, it is preferred that the nucleic acids specifically hybridize to a portion of the sequence set forth in SEQ ID NO: 32. Specific hybridization is herein defined as hybridization under standard stringent hybridization conditions. To identify and isolate other mammalian VEGF-C genes specifically, nucleic acid probes preferably are selected such that they fail to hybridize to genes related to VEGF-C (*e.g.*, fail to hybridize to human VEGF or to human VEGF-B genes).

Thus, the invention comprehends polynucleotides comprising at least about 16 nucleotides wherein the polynucleotides are capable of specifically hybridizing to a gene encoding VEGF-C, *e.g.*, a human gene. The specificity of hybridization ensures that a polynucleotide of the invention is able to hybridize to a nucleic acid encoding a VEGF-C under hybridization conditions that do not support hybridization of the polynucleotide to nucleic acids encoding, *e.g.*, VEGF or VEGF-B. In one embodiment, polynucleotides of at least about 16 nucleotides, and preferably at least about 20 nucleotides, are selected as continuous nucleotide sequences found in SEQ ID NO: 32 or the complement of the nucleotide sequence set forth in SEQ ID NO: 32.

Additional aspects of the invention include vectors which comprise nucleic acids of the invention; and host cells transformed or transfected with nucleic acids or vectors of the invention. Preferred vectors of the invention are expression vectors wherein nucleic acids of the invention are operatively connected to appropriate promoters and other control sequences, such that appropriate host cells transformed or transfected with the vectors are capable of expressing the Flt4 ligand. A preferred vector of the invention is plasmid pFLT4-L, having ATCC accession no. 97231. Such vectors and host cells are useful for recombinantly producing VEGF-C polypeptides.

The invention further includes a method of making polypeptides of the invention. In a preferred method, a nucleic acid or vector of the invention is expressed in a host cell, and a polypeptide of the invention is purified from the host cell or the host cell's growth medium.

In a related embodiment, the invention includes a method of making a polypeptide capable of specifically binding to Flt4 receptor tyrosine kinase, comprising the steps of: (a) transforming or transfecting a host cell

with a nucleic acid of the invention; (b) cultivating the host cell to express the nucleic acid; and (c) purifying a polypeptide capable of specifically binding to Flt4 receptor tyrosine kinase from the host cell or from the host cell's growth media.

The invention also is intended to include purified and isolated polypeptide ligands of Flt4 produced by methods of the invention. In one preferred embodiment, the invention includes a human VEGF-C polypeptide or biologically active fragment thereof that is substantially free of other human polypeptides.

In another aspect, the invention includes an antibody which is specifically reactive with polypeptides of the invention, or with polypeptides multimers of the invention. Antibodies, both monoclonal and polyclonal, may be made against a polypeptide of the invention according to standard techniques in the art. Such antibodies may be used in diagnostic applications to monitor angiogenesis, vascularization, lymphatic vessels and their disease states, wound healing, or certain tumor cells, hematopoietic, or leukemia cells. The antibodies also may be used to block the ligand from activating the Flt4 receptor.

Ligands according to the invention may be labeled with a detectable label and used to identify their corresponding receptors *in situ*. Labeled Flt4 ligand and anti-Flt4 ligand antibodies may be used as imaging agents in the detection of lymphatic vessels, high endothelial venules and their disease states, and Flt4 receptors expressed in histochemical tissue sections. The ligand or antibody may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, echogenic, or radioactive agent for imaging. Other, non-radioactive labels, such as biotin and avidin, may also be used.

A related aspect of the invention is a method for the detection of specific cells, *e.g.*, endothelial

cells. These cells may be found *in vivo*, or in *ex vivo* biological tissue samples. The method of detection comprises the steps of exposing a biological tissue comprising, *e.g.*, endothelial cells, to a polypeptide according to the invention which is capable of binding to Flt4 receptor tyrosine kinase, under conditions wherein the polypeptide binds to the cells, optionally washing the biological tissue, and detecting the polypeptide bound to the cells in the biological tissue, thereby detecting the cells.

The present invention also provides diagnostic and clinical applications for claimed ligands. In a preferred embodiment, Flt4 ligands or precursors are used to accelerate angiogenesis, *e.g.*, during wound healing, or to promote the endothelial functions of lymphatic vessels. A utility for VEGF-C is suggested as an inducer of angiogenesis also in tissue transplantation, in eye diseases, in the formation of collateral vessels around arterial stenoses and into injured tissues after infarction. Polypeptides according to the invention may be administered in any suitable manner using an appropriate pharmaceutically-acceptable vehicle, e.g., a pharmaceutically-acceptable diluent, adjuvant, excipient or carrier. VEGF-C polypeptides also may be used to quantify future metastatic risk by assaying biopsy material for the presence of active receptors or ligands in a binding assay or kit using detectably-labeled VEGF-C. An Flt4 ligand according to the invention also may be used to promote re-growth or permeability of lymphatic vessels in, for example, organ transplant patients. In addition, an Flt4 ligand may be used to mitigate the loss of axillary lymphatic vessels following surgical interventions in the treatment of cancer (e.g., breast cancer). Ligands according to the invention also may be used to treat or prevent inflammation, edema, aplasia of the lymphatic vessels, lymphatic obstruction, elephantiasis, and Milroy's disease. Finally, Flt4

ligands may be used to stimulate lymphocyte production and maturation, and to promote or inhibit trafficking of leukocytes between tissues and lymphatic vessels or to affect migration in and out of the thymus.

The invention includes a method of screening for an endothelial cell disorder in a mammalian subject. The method comprises providing a sample of endothelial cells from the subject, contacting the sample of endothelial cells with a polypeptide according to claim 4, determining the growth rate of the cells, and correlating the growth rate with a disorder. In a preferred embodiment, the endothelial cells are lymphatic endothelial cells. In another preferred embodiment, the mammalian subject is a human being and the endothelial cells are human cells. In yet another preferred embodiment, the disorder is a vessel disorder, *e.g.*, a lymphatic vessel disorder, such as the loss of lymphatic vessels through surgery or the reduction in function of existing lymphatic vessels due to blockages. In another embodiment, the endothelial cells are contacted with the polypeptide *in vitro*. The growth rate determined in the method is the rate of cell division per unit time, determined by any one of a number of techniques known in the art. The correlation of the growth rate with a disorder can involve a positive or negative correlation, *e.g.*, whether the polypeptide has Flt4 ligand activity or is an inhibitor of such activity, as described below.

Inhibitors of the Flt4 ligand may be used to control endothelial cell proliferation, lymphangiomas, and metastatic cancer. For example, such inhibitors may be used to arrest metastatic growth or spread, or to control other aspects of endothelial cell expression and growth. Inhibitors include antibodies, antisense oligonucleotides, and polypeptides which block the Flt4 receptor, all of which are intended as aspects of the invention.

In another embodiment, the invention provides a

method for modulating the growth of endothelial cells in a mammalian subject comprising the steps of exposing mammalian endothelial cells to a polypeptide according to the invention in an amount effective to modulate the growth of the mammalian endothelial cells. In one embodiment, the modulation of growth is effected by using a polypeptide capable of stimulating tyrosine phosphorylation of Flt4 receptor tyrosine kinase in a host cell expressing the Flt4 receptor tyrosine kinase. In modulating the growth of endothelial cells, the invention contemplates the modulation of endothelial cell-related disorders. Endothelial cell disorders contemplated by the invention include, but are not limited to, physical loss of lymphatic vessels (*e.g.*, surgical removal of axillary lymph tissue), lymphatic vessel occlusion (*e.g.*, elephantiasis), and lymphangiomas. In a preferred embodiment, the subject, and endothelial cells, are human. The endothelial cells may be provided *in vitro* or *in vivo*, and they may be contained in a tissue graft. An effective amount of a polypeptide is defined herein as that amount of polypeptide empirically determined to be necessary to achieve a reproducible change in cell growth rate (as determined by microscopic or macroscopic visualization and estimation of cell doubling time, or nucleic acid synthesis assays), as would be understood by one of ordinary skill in the art.

The present invention also provides methods for using the claimed nucleic acids (*i.e.*, polynucleotides) in screening for endothelial cell disorders. In a preferred embodiment, the invention provides a method for screening an endothelial cell disorder in a mammalian subject comprising the steps of providing a sample of endothelial cell nucleic acids from the subject, contacting the sample of endothelial cell nucleic acids with a polynucleotide of the invention which is capable of hybridizing to a gene encoding VEGF-C (and preferably

capable of hybridizing to VEGF-C mRNA), determining the level of hybridization between the endothelial cell nucleic acids and the polynucleotide, and correlating the level of hybridization with a disorder. A preferred mammalian subject, and source of endothelial cell nucleic acids, is a human. The disorders contemplated by the method of screening with polynucleotides include, but are not limited to, vessel disorders such as the aforementioned lymphatic vessel disorders, and hypoxia.

Purified and isolated polynucleotides encoding other (non-human) VEGF-C forms also are aspects of the invention, as are the polypeptides encoded thereby, and antibodies that are specifically immunoreactive with the non-human VEGF-C variants. Preferred non-human forms of VEGF-C are forms derived from other vertebrate species, including avian and mammalian species. Mammalian forms are highly preferred. Thus, the invention includes a purified and isolated mammalian VEGF-C polypeptide, and also a purified and isolated polynucleotide encoding such a polypeptide.

In one embodiment, the invention includes a purified and isolated polypeptide having the amino acid sequence of residues 1 to 415 of SEQ ID NO: 41, which sequence corresponds to a putative mouse VEGF-C precursor. The putative mouse VEGF-C precursor is believed to be processed into a mature mouse VEGF-C in a manner analogous to the processing of the human prepro-polypeptide. Thus, in a related aspect, the invention includes a purified and isolated polypeptide capable of specifically binding to an Flt4 receptor tyrosine kinase (e.g., a human or mouse Flt-4 receptor tyrosine kinase), the polypeptide comprising a fragment of the purified and isolated polypeptide having the amino acid sequence of residues 1 to 415 of SEQ ID NO: 41, the fragment being capable of specifically binding to the Flt4 receptor tyrosine kinase. The invention further includes multimers of the foregoing polypeptides and purified and

isolated nucleic acids encoding the foregoing polypeptides, such as a nucleic acid comprising all or a portion of the sequence shown in SEQ ID NO: 40.

In another embodiment, the invention includes a purified and isolated quail VEGF-C polypeptide, biologically active fragments and multimers thereof, and polynucleotides encoding the foregoing polypeptides.

In yet another embodiment, the invention includes a DNA comprising a VEGF-C promoter, that is capable of promoting expression of a VEGF-C gene or another operatively-linked, protein-encoding gene in native host cells, under conditions wherein VEGF-C is expressed in such cells.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 schematically depicts major endothelial cell receptor tyrosine kinases and growth factors involved in vasculogenesis and angiogenesis. Major structural domains are depicted, including immunoglobulin-like domains (IGH), epidermal growth factor homology domains (EGFH), fibronectin type III domains (FNIII), transmembrane (TM) and juxtamembrane (JM) domains, tyrosine kinase (TK1, TK2) domains, kinase insert domains (KI), and carboxy-terminal domains (CT).

Figure 2 schematically depicts the construction of the pLTRFlt41 expression vector.

Figure 3 schematically depicts the construction of the baculovirus vector encoding a secreted soluble Flt4 extracellular domain (Flt4EC).

Figure 4 shows results of stimulation of Flt4 autophosphorylation by conditioned medium from PC-3 cell cultures.

Figures 5A, 5B, and 5C show that the major tyrosyl phosphorylated polypeptide of Flt4-transfected cells stimulated with PC-3 conditioned medium is the 125 kD Flt4 polypeptide (VEGFR-3), and also that the Flt4 stimulating activity is not adsorbed to heparin-

sepharose.

Figure 6 shows Western immunoblotting analysis of the Flt4 ligand activity isolated from PC-3 conditioned medium.

Figure 7 shows results of gel electrophoresis of chromatographic fractions from the affinity purification of Flt4 ligand (VEGF-C) isolated from concentrated PC-3 conditioned medium.

Figure 8 depicts the amino acid sequences of human, murine, and quail VEGF-C polypeptides, aligned to show similarity. Residues conserved in all three species are depicted in bold.

Figure 9 schematically depicts the cloning and structure of the Flt4 ligand, VEGF-C. The VEGF homologous region (dark shaded box) and amino and carboxyl terminal propeptides (light shaded and unshaded boxes, respectively) as well as putative signal sequence (ss) are depicted between 5′ and 3′ untranslated (ut) nucleic acid regions. The cleavage sites for the signal sequence and the amino and carboxyl terminal propeptides are indicated with triangles.

Figure 10 shows a comparison of the deduced amino acid sequences of PDGF-A, -B, PlGF-1, VEGF-B_{167}, VEGF165, and Flt4 ligand (VEGF-C).

Figure 11 depicts the exon-intron organization of the human VEGF-C gene. Seven exons are depicted as open boxes, with exon size depicted in base pairs. Introns are depicted as lines, with intron size (base pairs) depicted above the lines. 5′ and 3′ untranslated sequences of a putative 2.4 kb mature mRNA are depicted as shaded boxes. The location of genomic clones used to characterize the VEGF-C gene are depicted below the map of the gene.

Figure 12 shows Northern blotting analysis of the genes encoding VEGF, VEGF-B, AND VEGF-C (indicated by "FLT4-L") in two human tumor cell lines and in brain tissue.

Figure 13A is an autoradiograph showing recombinant VEGF-C isolated following a pulse-chase experiment and electrophoresed via SDS-PAGE under reducing conditions.

Figure 13B is a photograph of polyacrylamide gel showing that recombinant VEGF-C forms are disulfide-linked in nonreducing conditions.

Figure 14A and 14B depict Western blots showing that VEGF-C stimulates autophosphorylation of VEGFR-2 (KDR) but has no effect on PDGFR-β phosphorylation.

Figure 15A shows that VEGF-C stimulates endothelial cell migration in a three-dimensional collagen gel assay.

Figure 16A shows the expression of VEGF-C mRNA in human adult tissues.

Figure 16B shows the expression of VEGF, VEGF-B, and VEGF-C in selected human fetal tissues.

Figure 17 depicts the genomic structure of the human and murine VEGF-C genes. Sequences of exon-intron junctions are depicted together with exon and intron lengths. Intron sequences are depicted in lower case letters. Nucleotides of the open reading frame observed in VEGF-C cDNAs are indicated as upper case letters in triplets (corresponding to the codons encoded at the junctions).

Figure 18 presents a schematic illustration of VEGF-C processing, including the major forms of VEGF-C.

Figure 19 depicts autoradiograms from a pulse-chase immunoprecipitation experiment wherein cells transfected with a VEGF-C expression vector (VEGF-C) and mock transfected cells (M) were pulse-labeled with radioactive amino acids and chased for varying lengths of time.

Figure 20 is a schematic map of the K14-VEGF-C vector construct.

Figures 21A-C depict electrophoretic fractionations of the various forms of recombinant VEGF-C

produced by transfected 293 EBNA cells. Figure 21B depicts the electrophoretic fractionation, under non-reducing conditions, of polypeptides produced from mock (M) transfected cells, cells transfected with wild type (wt) VEGF-C cDNA, and cells transfected with a cDNA variant encoding VEGF-C-R102S. Each of the bands identified in Figure 21B was excised and electrophoretically fractionated in a separate lane under reducing conditions. Fractionation of bands corresponding to wt VEGF-C are depicted in Figure 21A; fractionation of bands corresponding to the R102S variant are depicted in Figure 21C.

Figures 22A-B depict the forms and sizes of wild type and mutant recombinant VEGF-Cs, as revealed by non-reducing gel electrophoresis. Figure 22A shows the VEGF-C forms secreted into the media; Figure 22B shows the VEGF-C forms retained by the cells. Mock (M) transfected cells served as a control.

Figures 23A-B present a comparison of the pattern of immunoprecipitated, labelled VEGF-C forms using antisera 882 and antisera 905. Adjacent lanes contain immunoprecipitates that were (lanes marked +) or were not (lanes marked -) subjected to reduction and alkylation.

Figures 24A-B present Northern blots of total RNA isolated from cells grown in the presence or absence of interleukin-1 (IL-1) and/or dexamethasone (DEX) for the indicated times. For Figure 24B, the Northern blot was probed with radiolabeled DNA from a VEGF 581 bp cDNA covering bps 57-638 (Genbank Acc. No. X15997), and a human VEGF-B_{167} cDNA fragment (nucleotides 1/382, Genbank Acc. No. U48800). For Figure 24A, the Northern blot was probed with radiolabeled DNA from a human full-length VEGF-C cDNA (Genbank Acc. No. X94216). 18S and 28S- rRNA markers.

Figure 25 shows VEGF-C expression in *P. pastoris* cultures transfected with a VEGF-C cDNA, with

vector alone, or mock- (M) transfected, following induction with methanol for various periods of time as indicated. About 10 μl of medium was analyzed by gel electrophoresis followed by Western blotting and detection with anti-VEGF-C antiserum.

Figure 26 depicts the results of a Western blot wherein NIH 3T3 cells expressing VEGFR-3 (Flt4), and PAE cells expressing VEGFR-2 (KDR), were stimulated with 5x concentrated medium from *Pichia* yeast transfected with a VEGF-C cDNA-containing vector (+), with a vector lacking an insert (-), or stimulated with the positive control vanadate. The stimulated cells were lysed and immunoprecipitated with VEGFR-specific antibodies, and the immunoprecipitates were blotted and probed with anti-phosphotyrosine antibodies.

Figures 27A-B present gel electrophoretograms of human VEGF-C (wt) and VEGF-C variants secreted (Figure 27A) or retained (Figure 27B) by the host 293 EBNA cells. Mock (M) transfected cells served as a control. Molecular weight markers are indicated on the left in kilodaltons (kD).

Figures 28A-B show Western blots of VEGFRs that were stimulated to autophosphorylate by wild type (wt) VEGF-C, as well as three VEGF-C polypeptide variants. Cell lysates (NIH 3T3 for VEGFR-3 and PAE for VEGFR-2) were subjected to receptor-specific antisera and the receptors were immunoprecipitated. Immunoprecipitates were then gel-fractionated and blotted for Western analyses. Western blots were probed with anti-phosphotyrosine antibodies.

Figures 29A-D are photomicrographs of hematoxylin-eosin stained sections of K14-VEGF-C transgenic and control mouse littermate tissues. Areas shown are from the dorsal skin and snout, as indicated. The white arrows show the endothelium-lined margin of the lacunae devoid of red cells.

Figure 30 presents a Northern blot of

polyadenylated RNA from the indicated tissues, hybridized with a pool of VEGF, VEGF-B_{167}, and VEGF-C probes. Estimated transcript sizes are shown on the right in kilobases (kb).

Figure 31 presents a comparison of the human and mouse VEGF-C amino acid sequences. The amino acid sequence of mouse VEGF-C is presented on the top line and differences in the human sequence are marked below it. The sequences have been labeled to depict the regions shown in Figure 9. The arrow indicates the putative cleavage site for the signal peptidase; BR3P motifs, as well as a CR/SC motif, are boxed; and conserved cysteine residues are marked in bold above the sequence. Arginine residue 158 is also marked in bold. The numbering refers to mouse VEGF-C residues.

Figure 32 presents SDS-PAGE-fractionated samples immunoprecipitated or affinity-purified from various ^{35}S-labeled media. In the left panel, control medium from Bosc23 cells containing vector only, medium from cells expressing human VEGF-C, and medium from cells expressing mouse VEGF-C were independently precipitated with human VEGFR-3-Extracellular Domain coupled to sepharose. In the right panel, similar conditioned media were subjected to precipitation with anti-VEGF-C antibodies. mwm: molecular weight markers; m- mouse; h- human; α- anti.

Figure 33 shows Western blots of gel-fractionated immunoprecipitates from lysates made from NIH 3T3 cells expressing VEGFR-3 or VEGFR-2, as indicated, that had been stimulated by contact with VEGF-C-containing lysates (or a vector control), as a measure of VEGF-C-induced receptor autophosphorylation. Western blots were probed with anti-phosphotyrosine (α-PTyr) or anti-receptor antisera (anti-VEGFR-3 and anti-VEGFR-2), as indicated. As a control, receptor autophosphorylation was induced by pervanadate treatment (VO4). The arrows and numbers refer to the apparent molecular weights of

the tyrosyl phosphorylated receptor polypeptide bands. bVEGF: human baculoviral VEGF-C protein; C-FGEVm: lysate from cells harboring a mouse VEGF-C cDNA cloned into the vector in an antisense orientation.

Figures 34A-D depict photomicrographs of *in situ* hybridizations revealing the expression of VEGF-C and VEGF-B mRNAs in a parasagittal section of a 12.5 day mouse embryo. Figure 34A: VEGF-C probe; j- jugular veins, mn- metanephros, m- mesenterium (arrowheads), vc- intervertebral vessels, lu- lung (arrowheads). Figure 34B: VEGF-B probe; h- heart, nasopharyngeal area (arrowheads). Figure 34C: VEGF-C sense strand probe serving as a control. Figure 34D: bright-field photomicrograph of the same field shown in Figure 34C.

Figures 35A-H depict sections of mouse embryos providing comparisons of VEGF-C and VEGFR-3 expression in the jugular vessels and the mesenteric area. Figures 35A and 35C show expression of VEGF-C transcripts in the mesenchyme around the large sac-like structures in the jugular area (arrowheads). Figures 35B and 35D show expression of VEGFR-3 transcripts in the jugular venous sacs. Figures 35E and 35G show VEGF-C mRNA distribution in the mesenteric region of a 14.5 day p.c. embryo, as well as around the gut. Figures 35F and 35H show VEGFR-3 mRNA in the mesenteric region of a 14.5 day embryo, as well as the gut area, developing lymphatic vessels, and venules.

Figures 36A-D depict photomicrographs showing FLT4 and VEGF-C *in situ* hybridization of the cephalic region of a 16-day p.c. mouse embryo. A section of the cephalic region hybridized with the Flt4 probe (Figure 36A) shows the developing snout, nasal structures and eyes. A more caudally located section shows hybridization with the VEGF-C probe (Figure 36B). The round structures on both sides in the upper part represent the developing molars. In the upper (dorsal) part on both sides of the midline, the caudal portion of the developing conchae are seen.

These structures also are shown in higher magnification darkfield (Figure 36C) and lightfield (Figure 36D) microscopy.

DETAILED DESCRIPTION OF THE INVENTION

Described herein is the isolation of a novel vascular endothelial growth factor and the cloning of a DNA encoding this novel growth factor from a cDNA library prepared from the human prostatic adenocarcinoma cell line PC-3. The isolated cDNA encodes a protein which is proteolytically processed and secreted to cell culture medium. The secreted protein, designated VEGF-C, binds to the extracellular domain of Flt4 and induces tyrosine autophosphorylation of Flt4 and VEGFR-2. VEGF-C also stimulates the migration of endothelial cells in collagen gel.

Data reported herein indicates that VEGF-C is expressed as a larger precursor which is cleaved to produce the ligand. A coexpressed region in some cases results from alternative splicing of RNA of the ligand gene. Such a co-expressed region may be a function of the particular expression system used to obtain the ligand. The skilled artisan understands that in recombinant production of proteins, additional sequence may be expressed along with a functional polypeptide depending upon the particular recombinant construct used to express the protein, and subsequently removed to obtain the desired ligand. In some cases the recombinant ligand can be made lacking certain residues of the endogenous/natural ligand. Moreover, it is well-known in that conservative replacements may be made in a protein which do not alter the function of the protein. Accordingly, it is anticipated that such alterations are within the scope of the invention. Moreover, it is anticipated that one or more VEGF-C precursors (the largest putative native secreted VEGF-C precursor having the complete amino acid sequence from residue 32 to

residue 419 of SEQ ID NO: 33) is capable of stimulating the Flt4 ligand without any further processing, in a manner similar to that in which VEGF stimulates its receptor in its unprocessed form after the secretion and concomitant release of the signal sequence.

Results reported herein show that Flt4 (VEGFR-3) transmits signals for VEGF-C. This conclusion is based on the specific binding of VEGF-C to recombinant Flt4EC (Flt4 extracellular domain) protein and the induction of VEGFR-3 autophosphorylation by medium from VEGF-C transfected cells. In contrast, neither VEGF nor PlGF showed specific binding to VEGFR-3 or induced its autophosphorylation.

As set forth in greater detail below, the putative prepro-VEGF-C has a deduced molecular mass of 46,883; a putative prepro-VEGF-C processing intermediate has an observed molecular weight of about 32 kD; and mature VEGF-C isolated form conditioned media has a molecular weight of about 23 kD as assessed by SDS-PAGE under reducing conditions. A major part of the difference in the observed molecular mass of the purified and recombinant VEGF-C and the deduced molecular mass of the prepro-VEGF-C encoded by the VEGF-C open reading frame (ORF) is attributable to proteolytic removal of sequences at the amino-terminal and carboxyl-terminal regions of the prepro-VEGF-C polypeptide. However, proteolytic cleavage of the putative 102 amino acid leader sequence is not believed to account for the entire difference between the deduced molecular mass of 46,883 and the observed mass of about 23 kD, because the deduced molecular weight of a polypeptide consisting of amino acids 103-419 of SEQ ID NO: 33 is 35,881 kD. Evidence indicates that a portion of the observed difference in molecular weights is attributable to proteolytic removal of amino acid residues in both the amino and carboxyl terminal regions of the VEGF-C precursor. Extrapolation from studies of the structure of PDGF (Heldin *et al.*,

*Growth Factors, 8:*245-52 (1993)) suggests that the region critical for receptor binding and activation by VEGF-C is contained within amino acids residues 104-213, which are found in the secreted form of the VEGF-C protein (*i.e.*, the form lacking the putative prepro leader sequence and some carboxyterminal sequences). The 23 kD polypeptide binding VEGFR-3 is likely to represent the VEGF-homologous domain. After biosynthesis, the nascent VEGF-C polypeptide may be glycosylated at three putative N-linked glycosylation sites identified in the deduced VEGF-C amino acid sequence. Polypeptides containing modifications, such as N-linked glycosylations, are intended as aspects of the invention.

The carboxyl terminal amino acid sequences, which increase the length of the VEGF-C polypeptide in comparison with other ligands of this family, show a pattern of spacing of cysteine residues reminiscent of the Balbiani ring 3 protein (BR3P) sequence (Dignam *et al., Gene, 88:*133-40 (1990); Paulsson *et al., J. Mol. Biol., 211:*331-49 (1990)). This novel C-terminal silk protein-like structural motif of VEGF-C may fold into an independent domain, which, on the basis of the considerations above, is at least partially cleaved off after biosynthesis. Interestingly, at least one cysteine motif of the BR3P type is also found in the carboxyl terminus of VEGF. In our experiments both the putative precursor and cleaved ligand were detected in the cell culture media, suggesting cleavage by cellular proteases. The determination of amino-terminal and carboxy-terminal sequences of VEGF-C isolates allows the identification of the proteolytic processing sites. The generation of antibodies against different parts of the pro-VEGF-C molecule allows the exact determination of the precursor-product relationship and ratio, their cellular distribution, and the kinetics of processing and secretion.

VEGF-C has a conserved pattern of eight

cysteine residues, which may participate in the formation of intra- and interchain disulfide bonds, creating an antiparallel dimeric biologically active molecule, similar to PDGF. Mutational analysis of the cysteine residues involved in the interchain disulfide bridges has shown that, in contrast to PDGF, VEGF dimers need to be held together by these covalent interactions in order to maintain biological activity. Disulfide linking of the VEGF-C polypeptide chains was evident in the analysis of VEGF-C in nonreducing conditions, although recombinant protein also contained ligand-active VEGF-C forms which lacked disulfide bonds between the polypeptides.

VEGFR-3, which distinguishes between VEGF and VEGF-C, is closely related in structure to VEGFR-1 and VEGFR-2. Finnerty *et al.*, *Oncogene, 8*:2293-98 (1993); Galland *et al.*, *Oncogene, 8*:1233-40 (1993); Pajusola *et al.*, *Cancer Res., 52*:5738-43 (1992). Besides VEGFR-3, VEGFR-2 tyrosine kinase also is activated in response to VEGF-C. VEGFR-2 mediated signals cause striking changes in the morphology, actin reorganization and membrane ruffling of porcine aortic endothelial cells overexpressing this receptor. In these cells, VEGFR-2 also mediated ligand-induced chemotaxis and mitogenicity. Waltenberger *et al.*, *J. Biol. Chem., 269*:26988-95 (1994). Similarly, the receptor chimera CSF-1R/VEGFR-3 was mitogenic when ectopically expressed in NIH 3T3 fibroblastic cells, but not in porcine aortic endothelial cells (Pajusola *et al.*, 1994). Consistent with such results, the bovine capillary endothelial (BCE) cells, which express VEGFR-2 mRNA but very little or no VEGFR-1 or VEGFR-3 mRNAs, showed enhanced migration when stimulated with VEGF-C. Light microscopy of the BCE cell cultures in collagen gel also suggested that VEGF-C stimulated the proliferation of these cells. The data thus indicate that the VEGF ligands and receptors show a great specificity in their signalling, which may be cell-type-dependent.

The expression pattern of the VEGFR-3 (Kaipainen *et al., Proc. Natl. Acad. Sci. (USA), 92*:3566-70 (1995)) suggests that VEGF-C may function in the formation of the venous and lymphatic vascular systems during embryogenesis. Constitutive expression of VEGF-C in adult tissues shown herein further suggests that this gene product also is involved in the maintenance of the differentiated functions of the lymphatic and certain venous endothelia where VEGFR-3 is expressed (Kaipainen *et al.*, 1995). Lymphatic capillaries do not have well-formed basal laminae and an interesting possibility remains that the silk-like BR3P motif is involved in producing a supramolecular structure which could regulate the availability of VEGF-C in tissues. However, as shown here, VEGF-C also activates VEGFR-2, which is abundant in proliferating endothelial cells of vascular sprouts and branching vessels of embryonic tissues, but not so abundant in adult tissues. Millauer *et al., Nature, 367*:576-78 (1993). These data have suggested that VEGFR-2 is a major regulator of vasculogenesis and angiogenesis. VEGF-C may thus have a unique effect on lymphatic endothelium and a more redundant function, shared with VEGF, in angiogenesis and possibly in regulating the permeability of several types of endothelia. Because VEGF-C stimulates VEGFR-2 and promotes endothelial migration, VEGF-C may be useful as an inducer of angiogenesis of blood and lymphatic vessels in wound healing, in tissue transplantation, in eye diseases, and in the formation of collateral vessels around arterial stenoses and into injured tissues after infarction.

Taken together, these results show an increased complexity of signalling in the vascular endothelium. They reinforce the concept that when organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases in several types of functionally and morphologically

distinct vessels. However, upon stimulation by suitable angiogenic stimuli, endothelial cells can re-enter the cell cycle, migrate, withdraw from the cell cycle and subsequently differentiate again to form new vessels that are functionally adapted to their tissue environment. This process of angiogenesis, concurrent with tissue development and regeneration, depends on the tightly controlled balance between positive and negative signals for endothelial cell proliferation, migration, differentiation and survival.

Previously-identified growth factors promoting angiogenesis include the fibroblast growth factors, hepatocyte growth factor/scatter factor, PDGF and TGF-α. (*See e.g.*, Folkman, *Nature Med.*, *1*:27-31 (1995); Friesel *et al.*, *FASEB J.*, *9*:919-25 (1995); Mustonen *et al.*, *J. Cell. Biol.*, *129*:895-98 (1995). However, VEGF has been the only growth factor relatively specific for endothelial cells. The newly identified factors VEGF-B [Olofsson *et al.*, *Proc. Natl. Acad. Sci.*, *93*:2578-81 (1996)] and VEGF-C thus increase our understanding of the complexity of the specific and redundant positive signals for endothelial cells involved in vasculogenesis, angiogenesis, permeability, and perhaps also other endothelial functions. Expression studies using Northern blotting show abundant VEGF-C expression in heart and skeletal muscle; other tissues, such as placenta, ovary, small intestine, thyroid gland, kidney, prostate, spleen, testis and large intestine also express this gene. Whereas PlGF is predominantly expressed in the placenta, the expression patterns of VEGF, VEGF-B and VEGF-C overlap in many tissues, which suggests that members of the VEGF family may form heterodimers and interact to exert their physiological functions.

Targeted mutagenesis leading to inactivation of the VEGF receptor loci in the mouse genome has shown that VEGFR-1 is necessary for the proper organization of endothelial cells forming the vascular endothelium, while

VEGFR-2 is necessary for the generation of both endothelial and hematopoietic cells. This suggests that the four genes of the VEGF family can be targets for mutations leading to vascular malformations or cardiovascular diseases.

The following Examples illustrate preferred embodiments of the invention, wherein the isolation, characterization, and function of Flt4 ligands and ligand-encoding nucleic acids according to the invention are shown.

EXAMPLE 1

Production of pLTRFlt41 expression vector

Construction of the LTR-Flt41 vector, encoding the long form of Flt4 receptor tyrosine kinase, is schematically shown in Fig. 2. The full-length Flt4s (Flt4 short form) cDNA (Genbank Accession No. X68203, SEQ ID NO: 36) was assembled by first subcloning the S2.5 fragment, reported in Pajusola *et al., Cancer Res., 52*:5738-5743 (1992), incorporated by reference herein, containing base pairs 56-2534 of the Flt4s into the *Eco*RI site of the pSP73 vector (Promega, Madison, WI).

Since cDNA libraries used for screening of Flt4 cDNAs did not contain the extreme 5′ protein-coding sequences, inverse PCR was used for the amplification of the 5′ end of Flt4 corresponding to the first 12 amino acid residues (MQRGAALCLRLW). Poly(A)$^+$ RNA was isolated from human HEL erythroleukemia cells and double-stranded cDNA, were synthesized using an Amersham cDNA Synthesis System Plus kit (Amersham Corp., Buckinghamshire, U.K.) and a gene-specific primer: 5′-TGTCCTCGCTGTCCTTGTCT-3′ (SEQ ID NO: 1), which was located 195 bp downstream of the 5′ end of clone S2.5. Double-stranded cDNA was treated with T4 DNA polymerase to blunt the ends and cDNA was purified by filtration with Centricon 100 filters (Amicon Inc., Beverly, MA). Circularization of the blunt-ended cDNA was performed by ligation in a total

volume of 150 microliters. The reaction mixture contained a standard ligation buffer, 5% PEG-8000, 1 mM DTT and 8 U of T4 DNA ligase (New England Biolabs, Beverly, MA). Ligation was carried out at 16°C for 16 hours. Fifteen microliters of this reaction mix were used in a standard PCR reaction (100 µl total volume) containing 100 ng of Flt4-specific primers introducing *Sac*I and *Pst*I restriction sites, and 1 unit of Taq DNA polymerase (Perkin Elmer Cetus). Two rounds of PCR were performed using 33 cycles per round (denaturation at 95°C for 1 minute, annealing at 55°C for 2 minutes, and elongation at 72°C for 4 minutes). The PCR mixture was treated sequentially with the *Sac*I and *Pst*I restriction enzymes, and after purification with MagicPCR Preps (Promega), DNA fragments were subcloned into the pGEM3Zf(+) vector for sequencing (Promega). The sequence corresponded to the 5′ end of the Flt4s cDNA clone deposited in the Genbank Database as Accession No. X68203.

The sequence encoding the first 12 amino acid residues was added to the expression construct by ligating an *Sph*I-digested PCR fragment amplified using reverse transcription-PCR of poly(A)$^+$ RNA isolated from the HEL cells. The forward primer had the following sequence: 5′-ACATGCATGC CACC**ATG**CAG CGGGGCGCCG CGCTGTGCCT GCGACTGTGG CTCTGCCTGG GACTCCTGGA-3′ (SEQ ID NO: 2) (*Sph*I site underlined, translational start codon marked in bold). The translation start codon is immediately downstream from an optimized Kozak consensus sequence. (Kozak, *Nucl. Acids Res., 15:* 8125-8148 (1987).) The reverse primer, 5′-ACATGCATGC CCCGCCGGT CATCC-3′ (SEQ ID NO: 3) (*Sph*I site underlined), to the 5′ end of the S2.5 fragment, thus replacing the unique *Sph*I fragment of the S2.5 plasmid. The resulting vector was digested with *Eco*RI and *Cla*I and ligated to a 138 bp PCR fragment amplified from the 0.6 kb *Eco*RI fragment (base pairs 3789 to 4416 in the Genbank X68203 sequence) which encodes the

3′ end of Flt4s shown in Figure 1 of Pajusola *et al.*, *Cancer Res.*, *52:*5738-5743 (1992), using the oligonucleotides 5′-CGGAATTCCC CATGACCCCA AC-3′ (SEQ ID NO: 4) (forward primer, EcoRI site underlined) and 5′-CCATCGATGG ATCCTACCTG AAGCCGCTTT CTT-3′ (SEQ ID NO: 5) (reverse primer, *Cla*I site underlined). The coding domain was completed by ligation of the 1.2 kb *Eco*RI fragment (base pairs 2535-3789 of the sequence found in Gen Bank Acc. No. X68203) into the above construct. The complete cDNA was subcloned as a *Hin*dIII-*Cla*I(blunted) fragment (this *Cla*I site was also included in the 3′ primer used to construct the 3′ end of the coding sequence) to the pLTRpoly expression vector reported in Mäkelä *et al.*, *Gene, 118:* 293-294 (1992) (Genbank accession number X60280, SEQ ID NO: 37), incorporated by reference herein, using its *Hin*dIII-*Acc*I(blunted) restriction sites.

The long form of Flt4 (Flt41) was produced by replacing the 3′-end of the short form as follows: The 3′ region of the Flt41 cDNA was PCR-amplified using a gene-specific oligonucleotide (SEQ ID NO: 7, see below) and a pGEM 3Z vector-specific (SP6 promoter) oligonucleotide 5′-ATTTAGGTGACACTATA-3′ (SEQ ID NO: 6) as reverse and forward primers, respectively. The template for PCR was an Flt41 cDNA clone containing a 495 bp *Eco*RI fragment extending downstream of the *Eco*RI site at nucleotide 3789 of the Genbank X68203 sequence (the sequence downstream of this *Eco*RI site is deposited as the Flt4 long form 3′ sequence having Genbank accession number S66407 (SEQ ID NO: 38)). The gene-specific oligonucleotide contains a *Bam*HI restriction site located right after the end of the coding region and has the following sequence: 5′-CCATCGATGGATCCCGATGCTGCTTAGTAGCTGT-3′ (SEQ ID NO: 7) (*Bam*HI site is underlined). The PCR product was digested with *Eco*RI and *Bam*HI and transferred in frame to the LTRFlt4s vector fragment from which the coding sequences downstream of the EcoRI site at base pair 2535 (see

sequence X68203) had been removed by *Eco*RI-*Bam*HI digestion. The resulting clone was designated pLTRFlt41. Again, the coding domain was completed by ligation of the 1.2 kb *Eco*RI fragment (base pairs 2535-3789 of sequence X68203) back into the resulting construct.

EXAMPLE 2

Production and analysis of Flt41 transfected cells

NIH 3T3 cells (60 % confluent) were co-transfected with 5 micrograms of the pLTRFlt41 construct and 0.25 micrograms of the pSV2neo vector containing the neomycin phosphotransferase gene (Southern *et al., J. Mol. Appl. Genet., 1*:327 (1982)), using the DOTAP liposome-based transfection reagents (Boehringer-Mannheim, Mannheim, Germany). One day after transfection, the cells were transferred into selection media containing 0.5 mg/ml geneticin (GIBCO, Grand Island, N.Y.). Colonies of geneticin-resistant cells were isolated and analyzed for expression of the Flt4 proteins. Cells were lysed in boiling lysis buffer containing 3.3% SDS 125 mM Tris, pH 6.8. Protein concentrations of the samples were measured by the BCA method (Pierce, Rockford, IL). About 50 micrograms of protein from each lysate were analyzed for the presence of Flt4 by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using antisera against the carboxyl terminus of Flt4. Signals on Western blots were revealed using the ECL method (Amersham).

For production of anti-Flt4 antiserum, the Flt4 cDNA fragment encoding the 40 carboxy-terminal amino acid residues of the short form: NH2-PMTPTTYKG SVDNQTDSGM VLASEEFEQI ESRHRQESGFR-COOH (SEQ ID NO: 8) was cloned as a 657 bp *Eco*RI-fragment into the pGEX-1λT bacterial expression vector (Pharmacia-LKB, Inc., Uppsala, Sweden) in frame with the glutathione-S-transferase coding region. The resultant GST-Flt4S fusion protein was

produced in *E. coli* and purified by affinity chromatography using a glutathione-Sepharose 4B column. The purified protein was lyophilized, dissolved in phosphate-buffered saline (PBS), mixed with Freund's adjuvant and used for immunization of rabbits at bi-weekly intervals using methods standard in the art (Harlow *et al., Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1988)). Antisera were used, after the fourth booster immunization, for immunoprecipitation of Flt4 from transfected cells. Cell clones expressing Flt4 were also used for ligand stimulation analysis.

EXAMPLE 3

Construction of a Flt4 EC baculovirus vector and expression and purification of its product

The construction of an Flt4 extracellular domain (EC) baculovirus vector is schematically depicted in Fig. 3. The Flt4-encoding cDNA was prepared in both a long form and a short form, each being incorporated in a vector under control of the Moloney murine leukemia virus LTR promoter. The nucleotide sequence of the short form of the Flt4 receptor is available from the Genbank database as Accession No. X68203 and the specific 3' segment of the long form cDNA is available under GenBank Accession No. S66407.

The ends of a cDNA segment encoding the Flt4 extracellular domain (EC) were modified as follows: The 3' end of the Flt4 cDNA (Genbank Accession Number X68203) extracellular domain sequence was amplified using primer 1116 (5'-CTGGAGTCGACTTGGCGGACT-3'; SEQ ID NO: 9, *Sal*I site underlined) and primer 1315 (5'-CGCGGATCCCTAGTGATGGTGATGGTGATGTCTACCTTCGATCATGCTGCCCTTATCCTC-3'; (SEQ ID NO: 10, *Bam*HI site underlined). The sequence at the 5' end of primer 1315 is not complementary to the Flt4 coding region. Inspection of the sequence that is complementary to this region of

primer 1315 reveals in a 5′ to 3′ order, a stop codon, six contiguous histidine codons (for subsequent chromatographic purification of the encoded polypeptide using a Ni-NTA column; Qiagen, Hilden, Germany), and an added *Bam*HI site. The amplified fragment was digested with *Sal*I and *Bam*HI and used to replace a unique *Sal*I-*Bam*HI fragment in the LTRFlt4 vector shown in Fig. 3. The *Sal*I-*Bam*HI fragment that was replaced encodes the Flt4 transmembrane and cytoplasmic domains. The result was a modified LTRFlt4 vector.

The 5′ end without the Flt4 signal sequence encoding region was amplified by PCR using the primer 1335 (5′-CCCAAGCTTGGATCCAAGTGGCTACTCCATGACC-3′; (SEQ ID NO: 11) the primer contains added *Hin*dIII (AAGCTT) and *Bam*HI (GGATCC) restriction sites, which are underlined). The second primer used to amplify the region encoding the Flt4 signal sequence was primer 1332 5′-GTTGCCTGTGATGTGCACCA-3′; SEQ ID NO: 12). The amplified fragment was digested with *Hin*dIII and *Sph*I (the *Hin*dIII site (AAGCTT) is underlined in primer 1335 and the *Sph*I site is within the amplified region of the Flt4 cDNA). The resultant *Hin*dIII-*Sph*I fragment was used to replace a *Hin*dIII-*Sph*I fragment in the modified LTRFlt4 vector described immediately above (the *Hin*dIII site is in the 5′ junction of the Flt4 insert with the pLTRpoly portion of the vector, the *Sph*I site is in the Flt4 cDNA). The resultant Flt4EC insert was then ligated as a *Bam*HI fragment into the *Bam*HI site in the pVTBac plasmid described in Tessier *et al.*, *Gene 98*:177-183 (1991), incorporated herein by reference. The relative orientation of the insert was confirmed by partial sequencing so that the open reading frame of the signal sequence-encoding portion of the vector was adjacent to, and in frame with, the Flt4 coding region sequence. The Flt4EC construct was transfected together with baculovirus genomic DNA into SF-9 cells by lipofection. Recombinant virus was purified, amplified and used for

infection of High-Five cells (Invitrogen, San Diego, CA) using methods standard in the art. The Flt4 extracellular domain (Flt4EC) was purified from the culture medium of the infected High-Five cells using Ni-NTA affinity chromatography according to manufacturer's instructions (Qiagen) for binding and elution of the 6xHis tag encoded in the COOH-terminus of the recombinant Flt4 extracellular domain.

EXAMPLE 4

Isolation of an Flt4 Ligand from Conditioned Media

A human Flt4 ligand according to the invention was isolated from media conditioned by a PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) in Ham's F-12 Nutrient mixture (GIBCO) containing 7% fetal calf serum (FCS). The cells were grown according to the supplier's instructions. In order to prepare the conditioned media, confluent PC-3 cells were cultured for 7 days in Ham's F-12 Nutrient mixture (GIBCO) in the absence of fetal bovine serum (FBS). Medium was then cleared by centrifugation at 10,000 g for 20 minutes. The medium was then screened to determine its ability to induce tyrosine phosphorylation of Flt4 by exposure to NIH 3T3 cells which had been transfected with Flt4-encoding cDNA using the pLTRFlt41 vector. For receptor stimulation experiments, subconfluent NIH 3T3 cells were starved overnight in serum-free DMEM medium (GIBCO) containing 0.2% bovine serum albumin (BSA). The cells were stimulated with the conditioned media for 5 minutes, washed twice with cold PBS containing 100 micromolar vanadate, and lysed in RIPA buffer (10 mM Tris pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P40 (BDH, Poole, England), 0.1% SDS, 0.1 U/ml Aprotinin (Boehringer Mannheim), 1 mM vanadate) for receptor immunoprecipitation analysis. The lysates were centrifuged for 20 minutes at 15,000 x g. The supernatants were incubated for 2 hours on ice with 3

microliters of the antiserum against the Flt4 C-terminus described in Example 2. See also Pajusola *et al.*, *Oncogene*, *8*:2931-2937 (1993), incorporated by reference herein.

After a two hour incubation in the presence of anti-Flt4 antiserum, protein A-Sepharose (Pharmacia) was added and incubation was continued for 45 minutes with rotation. The immunoprecipitates were washed three times with the immunoprecipitation buffer and twice with 10 mM Tris, pH 7.5, before analysis by SDS-PAGE. Polypeptides were transferred to nitrocellulose and analyzed by Western blotting using Flt4- or phosphotyrosine-specific antisera and the ECL method (Amersham Corp.). Anti-phosphotyrosine monoclonal antibodies (anti-PTyr; PY20) were purchased from Transduction Laboratories (Lexington, Kentucky). In some cases, the filters were restained with a second antibody after stripping. The stripping of the filters was done for 30 minutes at 50°C in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, with occasional agitation.

As shown in Fig. 4, the PC-3 conditioned medium (PC-3CM), stimulated tyrosine phosphorylation of a 125 kD polypeptide when Flt4- expressing NIH 3T3 cells were treated with the indicated preparations of media, lysed, and the lysates were immunoprecipitated with anti-Flt4 antiserum followed by SDS-PAGE, Western blotting, and staining using anti-PTyr antibodies. The resulting band was weakly phosphorylated upon stimulation with unconcentrated PC-3 conditioned medium (lane 2). The 125 kD band comigrated with the tyrosine phosphorylated, processed form of the mature Flt4 from pervanadate-treated cells (compare lanes 2 and 7 of Fig. 4, see also Fig. 5A). Comigration was confirmed upon restaining with anti-Flt4 antibodies as is also shown in Fig. 5A (panel on the right). In order to show that the 125 kD polypeptide is not a non-specific component of the conditioned medium reactive with anti-phosphotyrosine

antibodies, 15 microliters of conditioned medium were separated by SDS-PAGE, blotted on nitrocellulose, and the blot was stained with anti-PTyr antibodies. No signal was obtained (Fig. 5B). Also, unconditioned medium failed to stimulate Flt4 phosphorylation, as shown in Fig. 4, lane 1.

Fig. 5C shows a comparison of the effects of PC-3 CM stimulation (+) on untransfected (lanes 4 and 5), FGFR-4-transfected (lanes 8 and 9) and Flt4-transfected NIH 3T3 cells (lanes 1-3, 6 and 7). These results indicate that neither untransfected NIH 3T3 cells nor NIH 3T3 cells transfected with FGFR-4 showed tyrosine phosphorylation of a protein of about 125 kD upon stimulation with the conditioned medium from PC-3 cells. Analysis of stimulation by PC-3 CM pretreated with Heparin-Sepharose CL-6B (Pharmacia) for 2 hours at room temperature (lane 3) showed that the Flt4 ligand does not bind to heparin.

As shown in Fig. 4, lane 3, stimulating activity was considerably increased when the PC-3 conditioned medium was concentrated four-fold using a Centricon-10 concentrator (Amicon). Fig. 4, lane 4, shows that pretreatment of the concentrated PC-3 conditioned medium with 50 microliters of the Flt4 extracellular domain coupled to CNBr-activated sepharose CL-4B (Pharmacia; about 1mg of Flt4EC domain/ml sepharose resin) completely abolished Flt4 tyrosine phosphorylation. Similar pretreatment of the conditioned medium with unsubstituted sepharose CL-4B did not affect stimulatory activity, as shown in Fig. 4, lane 5. Also, the flow through obtained after concentration, which contained proteins of less than 10,000 molecular weight, did not stimulate Flt4 phosphorylation, as shown in Fig. 4, lane 6.

In another experiment, a comparison of Flt4 autophosphorylation in transformed NIH 3T3 cells expressing LTRFlt41 was conducted, using unconditioned

medium, medium from PC-3 cells expressing the Flt4 ligand, or unconditioned medium containing either 50 ng/ml of VEGF165 or 50 ng/ml of PlGF-1. The cells were lysed, immunoprecipitated using anti-Flt4 antiserum and analyzed by Western blotting using anti-phosphotyrosine antibodies. Only the PC-3 conditioned medium expressing the Flt4 ligand (lane Flt-4L) stimulated Flt4 autophosphorylation.

The foregoing data show that PC-3 cells produce a ligand which binds to the extracellular domain of Flt4 and activates this receptor.

EXAMPLE 5

Purification of the Flt4 Ligand

The ligand expressed by human PC-3 cells as characterized in Example 4 was purified and isolated using a recombinantly-produced Flt4 extracellular domain (Flt4EC) in affinity chromatography.

Two harvests of serum-free conditioned medium, comprising a total of 8 liters, were collected from 500 confluent 15 cm diameter culture dishes containing confluent layers of PC-3 cells. The conditioned medium was clarified by centrifugation at 10,000 x g and concentrated 80-fold using an Ultrasette Tangential Flow Device (Filtron, Northborough, MA) with a 10 kD cutoff Omega Ultrafiltration membrane according to the manufacturer's instructions. Recombinant Flt4 extracellular domain was expressed in a recombinant baculovirus cell system and purified by affinity chromatography on Ni-agarose (Ni-NTA affinity column obtained from Qiagen). The purified extracellular domain was coupled to CNBr-activated Sepharose CL-4B at a concentration of 5 mg/ml and used as an affinity matrix for ligand affinity chromatography.

Concentrated conditioned medium was incubated with 2 ml of the recombinant Flt4 extracellular domain-Sepharose affinity matrix in a rolling tube at room

temperature for 3 hours. All subsequent purification steps were at +4 °C. The affinity matrix was then transferred to a column with an inner diameter of 15 mm and washed successively with 100 ml of PBS and 50 ml of 10 mM Na-phosphate buffer (pH 6.8). Bound material was eluted step-wise with 100 mM glycine-HCl, successive 6 ml elutions having pHs of 4.0, 2.4, and 1.9. Several 2 ml fractions of the eluate were collected in tubes containing 0.5 ml 1 M Na-phosphate (pH 8.0). Fractions were mixed immediately and dialyzed in 1 mM Tris-HCl (pH 7.5). Aliquots of 75 µl each were analyzed for their ability to stimulate tyrosine phosphorylation of Flt4. The ultrafiltrate, 100 µl aliquots of the concentrated conditioned medium before and after ligand affinity chromatography, as well as 15-fold concentrated fractions of material released from the Flt4 extracellular domain-Sepharose matrix during the washings were also analyzed for their ability to stimulate Flt4 tyrosine phosphorylation.

As shown in Fig. 6, lane 3, the concentrated conditioned medium induced prominent tyrosine phosphorylation of Flt4 in transfected NIH 3T3 cells overexpressing Flt4. This activity was not observed in conditioned medium taken after medium was exposed to the Flt4 Sepharose affinity matrix described above (Fig. 6, lane 4). The specifically-bound Flt4-stimulating material was retained on the affinity matrix after washing in PBS, 10 mM Na-phosphate buffer (pH 6.8), and at pH 4.0 (Fig. 6, lanes 5-7, respectively), and it was eluted in the first two 2 ml aliquots at pH 2.4 (lanes 8 and 9). A further decrease of the pH of the elution buffer did not cause release of additional Flt4-stimulating material (Fig. 6, lane 11). Fig. 6, lane 1 depicts a control wherein Flt4-expressing cells were treated with unconditioned medium; lane 2 depicts the results following treatment of Flt4-expressing cells with the ultrafiltrate fraction of conditioned medium

containing polypeptides of less than 10 kD molecular weight.

Small aliquots of the chromatographic fractions were concentrated in a SpeedVac concentrator (Savant, Farmingdale, N.Y.) and subjected to SDS-PAGE under reducing conditions with subsequent silver staining of the gel, a standard technique in the art. As shown in Fig. 7, the major polypeptide, having a molecular weight of approximately 23 kD (reducing conditions), was detected in the fractions containing Flt4 stimulating activity (corresponding to lanes 8 and 9 in Fig. 6). That polypeptide was not found in the other chromatographic fractions. On the other hand, besides these bands and a very faint band having a 32 kD mobility, all other components detected in the two active fractions were also distributed in the starting material and in small amounts in the other washing and eluting steps after their concentration. Similar results were obtained in three independent affinity purifications, indicating that the 23 kD polypeptide specifically binds to Flt4 and induces tyrosine phosphorylation of Flt4.

Fractions containing the 23 kD polypeptide were combined, dried in a SpeedVac concentrator and subjected to SDS-PAGE in a 12.5% gel. The proteins from the gel were then electroblotted to Immobilon-P (PVDF) transfer membrane (Millipore, Marlborough, MA) and visualized by staining of the blot with Coomassie Blue R-250. The region containing only the stained 23 kD band was cut from the blot and subjected to N-terminal amino acid sequence analysis in a Prosite Protein Sequencing System (Applied Biosystems, Foster City, CA). The data were analyzed using a 610A Data Analysis System (Applied Biosystems). Analysis revealed a single N-terminal sequence of NH_2-XEETIKFAAAHYNTEILK-COOH (SEQ ID NO: 13).

EXAMPLE 6

Construction of PC-3 cell cDNA library in a eukaryotic expression vector

Human poly(A)$^+$ RNA was isolated from five 15 cm diameter dishes of confluent PC-3 cells by a single step method using oligo(dT) (Type III, Collaborative Biomedical Products, Becton-Dickinson Labware, Bedford, MA) cellulose affinity chromatography (Sambrook *et al.*, 1989). The yield was 70 micrograms. Six micrograms of the Poly(A)$^+$ RNA were used to prepare an oligo(dT)-primed cDNA library in the mammalian expression vector pcDNA I and the Librarian kit of Invitrogen according to the instructions included in the kit. The library was estimated to contain about 10^6 independent recombinants with an average insert size of approximately 1.8 kb.

EXAMPLE 7

Amplification of a unique nucleotide sequence encoding the Flt4 ligand amino terminus

Degenerate oligonucleotides were designed based on the N-terminal amino acid sequence of the isolated human Flt4 ligand and were used as primers in a polymerase chain reaction (PCR) to amplify cDNA encoding the Flt4 ligand from the PC-3 cDNA library. The overall strategy described in Examples 7 and 8 is schematically depicted in Fig. 9, where the different primers have been marked with arrows.

The PCR was carried out using 1 microgram of DNA from the amplified PC-3 cDNA library and a mixture of 48 sense-strand primers present in equal proportions, the primer sequences collectively comprising the sequence 5'-GCAGARGARACNATHAA-3' (SEQ ID NO: 14) (wherein R is A or G, N is A,G,C or T and H is A, C or T), encoding amino acid residues 2-6 (EETIK, SEQ ID NO: 15) and 384 antisense-strand primers present in equal proportions, the anti-sense strand primers collectively comprising the sequence 5'-GCAYTTNARDATYTCNGT-3' (SEQ ID NO: 16)

(wherein Y is C or T and D is A, G or T), corresponding to amino acid residues 14-18 (TEILK, SEQ ID NO: 17). Three extra nucleotides (GCA) were added to the 5'-terminus of each primer to increase annealing stability. Two successive PCR runs were carried out using 1 U per reaction of DynaZyme (F-500L, Finnzymes, Espoo, Finland), a thermostable DNA polymerase, in a buffer supplied by the manufacturer (10 mM Tris-HCl, pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton-X100), at an extension temperature of 72°C. The first PCR run was carried out for 43 cycles. The first three cycles were run at an annealing temperature of 33°C for 2 minutes, and the remaining cycles were run at 42°C for 1 minute.

The region of the gel containing a weak band of the expected size (57 bp) was cut out from the gel and eluted. The eluted material was reamplified for 30 cycles using the same primer pairs described above at 42°C for 1 minute. The amplified fragment was cloned into a pCR II vector (Invitrogen) using the TA cloning kit (Invitrogen) and sequenced using the radioactive dideoxynucleotide sequencing method of Sanger. Six clones were analyzed and all six clones contained the sequence encoding the expected peptide (amino acid residues 104-120 of the Flt4 ligand precursor). Nucleotide sequence spanning the region from the third nucleotide of codon 6 to the third nucleotide of codon 13 (the extension region) was identical in all six clones: 5'-ATTCGCTGCAGCACACTACAAC-3' (SEQ ID NO: 18) and thus represented an amplified product from the unique sequence encoding part of the amino terminus of the Flt4 ligand.

EXAMPLE 8

Amplification of the 5'-end of the cDNA encoding the Flt4 ligand

Based on the unique nucleotide sequence encoding the N-terminus of the isolated human Flt4 ligand, two pairs of nested primers were designed to

amplify, in two nested PCR reactions, the complete 5'-end of the corresponding cDNAs from one microgram of DNA of the above-described PC-3 cDNA library. First, amplification was performed with an equal mixture of 4 primers collectively defining the sequence 5'-TCNGTGTTGTAGTGTGCTG-3' (SEQ ID NO: 19), which is the antisense-strand primer corresponding to amino acid residues 9-15 (AAHYNTE, SEQ ID NO: 20), and sense-strand primer 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO: 21), corresponding to the T7 RNA promoter of the pcDNAI vector used for construction of the library. "Touchdown" PCR was used as disclosed in Don *et al.*, *Nucl. Acids Res.*, *19*:4008 (1991), incorporated by reference herein. The annealing temperature of the two first cycles was 62°C and subsequently the annealing temperature was decreased in every other cycle by 1°C until a final temperature of 53°C was reached, at which temperature 16 additional cycles were conducted. Annealing time was 1 minute and extension at each cycle was conducted at 72°C for 1 minute. Multiple amplified DNA fragments were obtained in the first reaction. The products of the first amplification (1 µl of a 1:100 dilution in water) were used in the second amplification reaction employing a pair of nested primers comprising an antisense-strand primer 5'-GTTGTAGTGTGCTGCAGCGAATTT-3'; SEQ ID NO: 22) encoding amino acid residues 6-13 (KFAAAHYN, SEQ ID NO: 23) of the Flt4 ligand, and a sense-strand primer (5'-TCACTATAGGGAGACCCAAGC-3'; SEQ ID NO: 24), corresponding to nucleotides 2179-2199 of the pcDNAI vector. The sequences of these sense and antisense primers overlapped with the 3' ends of the corresponding primers used in the first PCR. "Touchdown" PCR was carried out by decreasing the annealing temperature from 72°C to 66°C and continuing with 18 additional cycles at 66°C. The annealing time was 1 minute and extension at each cycle was carried out at 72°C for 2 minutes. One major product of about 220 bp and three minor products of about 270 bp, 150 bp, and 100 bp

were obtained.

The amplified fragment of approximately 220 bp was excised from an agarose gel, cloned into a pCRII vector using the TA cloning kit (Invitrogen), and sequenced. Three recombinant clones were analyzed and they contained the sequence 5′-TCACTATAGGGAGACCCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCC AGTGTGGTGGAATTCGACGAACTCATGACTGTACTCTACCCAGAATATTGGAAAATG TACAAGTGTCAGCTAAGGCAAGGAGGCTGGCAACATAACAGAGAACAGGCCAACCTC AACTCAAGGACAGAAGAGACTATAAAATTCGCTGCAGCACACTACAAC- 3′ (SEQ ID NO: 25). The beginning of the sequence represents the pcDNAI vector and the underlined sequence represents the amplified product of the 5′-end of the cDNA insert.

EXAMPLE 9

Amplification of the 3′-end of cDNA encoding the Flt4 ligand

Based upon the amplified 5′-sequence of the clones encoding the amino terminus of the 23 kD human Flt4 ligand, two pairs of non-overlapping nested primers were designed to amplify the 3′-portion of the Flt-4-ligand-encoding cDNA clones. The sense-strand primer 5′-ACAGAGAACAGGCCAACC-3′ (SEQ ID NO: 26), corresponding to nucleotides 152-169 of the amplified 5′-sequences of the Flt4 ligand (SEQ ID NO: 25), and antisense-strand primer 5′-TCTAGCATTTAGGTGACAC-3′ (SEQ ID NO: 27) corresponding to nucleotides 2311-2329 of the pcDNAI vector were used in a first "touchdown" PCR. The annealing temperature of the reaction was decreased 1°C every two cycles from 72°C to 52°C, at which temperature 15 additional cycles were carried out. The annealing time was 1 minute and extension at each cycle was carried out at 72°C for 3 minutes. DNA fragments of several sizes were obtained in the first amplification. Those products were diluted 1:200 in water and reamplified in PCR using the second pair of primers: 5′-AAGAGACTATAAAATTCGCTGCAGC-3′ (SEQ ID NO: 28) and 5′-CCCTCTAGATGCATGCTCGA-3′ (SEQ ID NO: 29) (antisense-strand primer corresponding to nucleotides

2279-2298 of the pcDNAI vector). Two DNA fragments were obtained, having sizes of 1350 bp and 570 bp. Those fragments were cloned into a pCRII vector and the inserts of the clones were sequenced. Both of these fragments were found to contain sequences encoding an amino acid sequence homologous to the VEGF sequence.

EXAMPLE 10

Screening the PC-3 cell cDNA library using the 5' PCR fragment of Flt4 ligand cDNA

A 219 bp 5'-terminal fragment of human Flt4 ligand cDNA was amplified by PCR using the 5' PCR fragment described above and primers 5'-GTTGTAGTGTGCTGCAGCGAATTT-3' (antisense-strand primer, SEQ ID NO: 30) and 5'-TCACTATAGGGAGACCCAAGC-3' (SEQ ID NO: 31) (sense-primer corresponding to nucleotides 2179-2199 of the pcDNAI vector). The amplified product was subjected to digestion with *Eco*RI (Boehringer Mannheim) to remove the portion of the DNA sequence amplified from the pcDNAI vector and the resulting 153 bp fragment encoding the 5' end of the Flt4 ligand was labeled with [^{32}P]-dCTP using the Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim). That fragment was used as a probe for hybridization screening of the amplified PC-3 cell cDNA library.

Filter replicas of the library were hybridized with the radioactively labeled probe at 42°C for 20 hours in a solution containing 50% formamide, 5x SSPE, 5x Denhardt's solution, 0.1% SDS and 0.1 mg/ml denatured salmon sperm DNA. Filters were washed twice in 1x SSC, 0.1% SDS for 30 minutes at room temperature, then twice for 30 minutes at 65°C and exposed overnight.

On the basis of autoradiography, 10 positive recombinant bacterial colonies hybridizing with the probe were chosen from the library. Plasmid DNA was purified from these colonies and analyzed by *Eco*RI and *Not*I digestion and agarose gel electrophoresis followed by

ethidium bromide staining. The ten plasmid clones were divided into three groups on the basis of the presence of insert sizes of approximately 1.7, 1.9 and 2.1 kb, respectively. Inserts of plasmids from each group were sequenced using the T7 oligonucleotide as a primer and walking primers for subsequent sequencing reactions.

Sequence analysis showed that all clones contain the open reading frame encoding the NH2-terminal sequence of the 23 kD human Flt4 ligand. Dideoxy sequencing was continued using walking primers in the downstream direction. A complete human cDNA sequence and deduced amino acid sequence from a 2 kb clone is set forth in SEQ ID NOs: 32 and 33, respectively. A putative cleavage site of a "prepro" leader sequence is located between residues 102 and 103 of SEQ ID NO: 33. When compared with sequences in the GenBank Database, the predicted protein product of this reading frame was found to be homologous with the predicted amino acid sequences of the PDGF/VEGF family of growth factors, as shown in Fig. 10.

Plasmid pFLT4-L, containing the 2.1 kb human cDNA clone in pcDNAI vector, has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 as accession number 97231.

EXAMPLE 11

Stimulation of Flt4 autophosphorylation by the protein product of the Flt4 ligand vector

The 2.1 kb human cDNA insert of plasmid pFlt4-L, which contains the open reading frame encoding the sequence shown in SEQ ID NOs: 32 and 33; human VEGF-C, see below), was cut out from the pcDNAI vector using *Hin*dIII and *Not*I restriction enzymes, isolated from a preparative agarose gel, and ligated to the corresponding sites in the pREP7 expression vector (Invitrogen). The pREP7 vector containing the pFlt4-L insert was transfected into 293-EBNA cells (Invitrogen) using the

calcium phosphate transfection method (Sambrook *et al.*, 1989). About 48 hours after transfection the medium of the transfected cells was changed to DMEM medium lacking fetal calf serum and incubated for 36 h. The conditioned medium was then collected, centrifuged at 5000 x g for 20 minutes, the supernatant was concentrated 5-fold using Centriprep 10 (Amicon) and used to stimulate NIH 3T3 cells expressing LTRFlt41 (the Flt4 receptor), as in Example 4. The cells were lysed, immunoprecipitated using anti-Flt4 antiserum and analyzed by Western blotting using anti-phosphotyrosine antibodies.

The conditioned medium from two different dishes of the transfected cells stimulated Flt4 autophosphorylation in comparison with the medium from mock-transfected cells, which gave only background levels of phosphorylation of the Flt4 receptor. When the concentrated conditioned medium was pre-absorbed with 20 microliters of a slurry of Flt4EC domain coupled to Sepharose (see example 4), no phosphorylation was obtained, showing that the activity responsible for Flt4 autophosphorylation was indeed the Flt4 ligand. Thus, these results demonstrate that an expression vector having an approximately 2.1 kb insert and containing an open reading frame as shown in SEQ ID NO: 32 is expressed as a biologically active Flt4 ligand (VEGF-C) in transfected cells. The sequence encoded by that open reading frame is shown in SEQ ID NO: 33.

The deduced molecular weight of a polypeptide consisting of the complete amino acid sequence in SEQ ID NO: 33 (residues 1 to 419) is 46,883. The deduced molecular weight of a polypeptide consisting of amino acid residues 103 to 419 of SEQ ID NO: 33 is 35,881. The Flt4 ligand purified from PC-3 cultures had an observed molecular weight of about 23 kD as assessed by SDS-PAGE under reducing conditions. Thus, it appears that the Flt4 ligand mRNA is translated into a precursor polypeptide, from which the mature ligand is derived by

proteolytic cleavage. Also, the Flt4 ligand may be glycosylated at three putative N-linked glycosylation sites conforming to the consensus which can be identified in the deduced Flt4 ligand amino acid sequence (N-residues underlined in Fig. 10).

The carboxyl terminal amino acid sequences, which increase the predicted molecular weight of the Flt4 ligand subunit in comparison with other ligands of this family, show a pattern of spacing of cysteine residues reminiscent of the Balbiani ring 3 protein (BR3P) sequence (Dignam *et al., Gene, 88*:133-140 (1990)), as depicted schematically in Fig. 9. Such a sequence may encode an independently folded domain present in a Flt4 ligand precursor and it may be involved, for example, in the regulation of secretion, solubility, stability, cell surface localization or activity of the Flt4 ligand. Interestingly, at least one cysteine motif of the BR3P type is also found in the VEGF carboxy terminal amino acid sequences.

Thus, the Flt4 ligand mRNA appears first to be translated into a precursor from the mRNA corresponding to the cDNA insert of plasmid FLT4-L, from which the mature ligand is derived by proteolytic cleavage. To define the mature Flt4 ligand polypeptide, one first expresses the cDNA clone (which is deposited in the pcDNAI expression vector) in cells, such as COS cells. One uses antibodies generated against encoded polypeptides, fragments thereof, or bacterial Flt4 fusion proteins, such as a GST-fusion protein, to raise antibodies against the VEGF-homologous domain and the amino- and carboxyl-terminal propeptides of Flt4 ligand. One then follows the biosynthesis and processing of the Flt4 ligand in the transfected cells by pulse-chase analysis using radioactive cysteine for labelling of the cells, immunoprecipitation and gel electrophoresis. Using antibodies against the three domains of the product encoded by the cDNA insert of plasmid FLT4-L, material

for radioactive or nonradioactive amino-terminal sequence analysis is isolated. The determination of the amino-terminal sequence of the mature VEGF-C polypeptide allows for identification of the amino-terminal proteolytic processing site. The determination of the amino-terminal sequence of the carboxyl-terminal propeptide will give the carboxyl-terminal processing site. This is confirmed by site-directed mutagenesis of the amino acid residues adjacent to the cleavage sites, which would prevent the cleavage.

The Flt4 ligand is further characterizeable by progressive 3′ deletions in the 3′ coding sequences of the Flt4 ligand precursor clone, introducing a stop codon resulting in carboxy-terminal truncations of its protein product. The activities of such truncated forms are assayed by, for example, studying Flt4 autophosphorylation induced by the truncated proteins when applied to cultures of cells, such as NIH 3T3 cells expressing LTRFlt4. By extrapolation from studies of the structure of the related platelet derived growth factor (PDGF, Heldin *et al.*, *Growth Factors, 8*:245-252 (1993)) one determines that the region critical for receptor activation by the Flt4 ligand is contained within the first approximately 180 amino acid residues of the secreted VEGF-C protein lacking the putative 102 amino acid prepro leader (SEQ ID NO: 33, residues 103-282), and apparently within the first approximately 120 amino acid residues (SEQ ID NO: 33, residues 103-223).

On the other hand, the difference between the molecular weights observed for the purified ligand and deduced from the open reading frame of the Flt4 ligand clone may be due to the fact that the soluble ligand was produced from an alternatively spliced mRNA which would also be present in the PC-3 cells, from which the isolated ligand was derived. To isolate such alternative cDNA clones one uses cDNA fragments of the deposited clone and PCR primers made according to the sequence

provided as well as techniques standard in the art to isolate or amplify alternative cDNAs from the PC-3 cell cDNA library. One may also amplify using reverse transcription (RT)-PCR directly from the PC-3 mRNA using the primers provided in the sequence of the cDNA insert of plasmid FLT4-L. Alternative cDNA sequences are determined from the resulting cDNA clones. One can also isolate genomic clones corresponding to the Flt4 ligand mRNA transcript from a human genomic DNA library using methods standard in the art and to sequence such clones or their subcloned fragments to reveal the corresponding exons. Alternative exons can then be identified by a number of methods standard in the art, such as heteroduplex analysis of cDNA and genomic DNA, which are subsequently characterized.

EXAMPLE 12

Expression of the Gene Encoding VEGF-C in Human Tumor Cell Lines

Expression of transcripts corresponding to the Flt4 ligand (VEGF-C) was analyzed by hybridization of Northern blots containing isolated poly(A)$^+$ RNA from HT-1080 and PC-3 human tumor cell lines. The probe was the radioactively labelled insert of the 2.1 kb cDNA clone (pFlt4-L/VEGF-C, specific activity 10^8-10^9 cpm/mg of DNA). The blot was hybridized overnight at 42°C using 50% formamide, 5x SSPE buffer, 2% SDS, 10 x Denhardt's solution, 100 mg/ml salmon sperm DNA and 1 x 10^6 cpm of the labelled probe/ml. The blot was washed at room temperature for 2 x 30 minutes in 2x SSC containing 0.05% SDS, and then for 2 x 20 minutes at 52°C in 0.1x SSC containing 0.1% SDS. The blot was then exposed at -70°C for three days using intensifying screens and Kodak XAR film. Both cell lines expressed an Flt4 ligand mRNA of about 2.4 kb, as well as VEGF and VEGF-B mRNAs (Fig. 12).

EXAMPLE 13

VEGF-C Chains Are Proteolytically Processed after Biosynthesis and Disulfide Linked

The predicted molecular mass of a secreted human VEGF-C polypeptide, as deduced from the VEGF-C open reading frame, is 46,883 kD, suggesting that VEGF-C mRNA may be first translated into a precursor, from which the ligands of 21/23 kD and 29/32 kD are derived by proteolytic cleavage.

This possibility was explored by metabolic labelling of 293 EBNA cells expressing VEGF-C. Initially, 293 EBNA cells were transfected with the VEGF-C construct. Expression products were labeled by the addition of 100 μCi/ml of Pro-mix™ L-[^{35}S] *in vitro* cell labelling mix ((containing ^{35}S-methionine and ^{35}S-cysteine) Amersham, Buckinghamshire, England) to the culture medium devoid of cysteine and methionine. After two hours, the cell layers were washed twice with PBS and the medium was then replaced with DMEM-0.2% BSA. After 1, 3, 6, 12 and 24 hours of subsequent incubation, the culture medium was collected, clarified by centrifugation, and concentrated, and human VEGF-C was bound to 30 μl of a slurry of Flt4EC-Sepharose overnight at +4°C, followed by three washes in PBS, two washes in 20 mM Tris-HCl (pH 7.5), alkylation, SDS-PAGE and autoradiography. Alkylation was carried out by treatment of the samples with 10mM 1,4 Dithiothreitol (Boehringer-Mannheim, Mannheim, Germany) for one hour at 25°C, and subsequently with 30 mM iodoacetamide (Fluka, Buchs, Switzerland).

These experiments demonstrated that a putative precursor polypeptide of 32 kD apparent molecular mass was bound to the Flt4EC affinity matrix from the conditioned medium of metabolically labelled cells transfected with the human VEGF-C expression vector (Fig. 13A), but not from mock (M) transfected cells. Increased amounts of a 23 kD receptor binding polypeptide

accumulated in the culture medium of VEGF-C transfected cells during a subsequent chase period of three hours, but not thereafter (lanes 2-4 and data not shown), suggesting that the 23 kD form is produced by proteolytic processing, which is incomplete, at least in the transiently transfected cells. The arrows in Fig. 13A indicate the 32 kD and 23 kD polypeptides of secreted VEGF-C. Subsequent experiments showed that the 32kD VEGF-C form contains two components migrating in the absence of alkylation as polypeptides of 29 and 32 kD (Figs. 21-23).

In a related experiment, human VEGF-C isolated using Flt4EC-Sepharose after a 4 h continuous metabolic labelling was analyzed by polyacrylamide gel electrophoresis in nonreducing conditions (Fig. 13B). Higher molecular mass forms were observed under nonreducing conditions, suggesting that the VEGF-C polypeptides can form disulfide-linked dimers and/or multimers (arrows in Fig. 13B).

EXAMPLE 14

Stimulation Of VEGFR-2 Autophosphorylation By VEGF-C

Conditioned medium (CM) from 293 EBNA cells transfected with the human VEGF-C vector also was used to stimulate porcine aortic endothelial (PAE) cells expressing VEGFR-2 (Kdr). Pajusola *et al.*, *Oncogene, 9:*3545-55 (1994); Waltenberger *et al.*, *J. Biol. Chem., 269:*26988-26995 (1994). The cells were lysed and immunoprecipitated using VEGFR-2 - specific antiserum (Waltenberger *et al.*, 1994).

PAE-KDR cells (Waltenberger *et al.*, 1994) were grown in Ham's F12 medium-10% fetal calf serum (FCS). Confluent NIH 3T3-Flt4 cells or PAE-KDR cells were starved overnight in DMEM or Ham's F12 medium, respectively, supplemented with 0.2% bovine serum albumin (BSA), and then incubated for 5 minutes with the analyzed media. Recombinant human VEGF (R&D Systems) and PDGF-BB,

functional as stimulating agents, were used as controls. The cells were washed twice with ice-cold Tris-Buffered Saline (TBS) containing 100 mM sodium orthovanadate and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 U/ml aprotinin and 1 mM sodium orthovanadate. The lysates were sonicated, clarified by centrifugation at 16,000 x g for 20 minutes and incubated for 3-6 hours on ice with 3-5 μl of antisera specific for Flt4 (Pajusola *et al.*, 1993), VEGFR-2 or PDGFR-β (Claesson-Welsh *et al., J. Biol. Chem., 264*:1742-1747 (1989); Waltenberger *et al.*, 1994). Immunoprecipitates were bound to protein A-Sepharose, washed three times with RIPA buffer containing 1mM PMSF, 1mM sodium orthovanadate, washed twice with 10 mM Tris-HCl (pH 7.4), and subjected to SDS-PAGE using a 7% gel. Polypeptides were transferred to nitrocellulose by Western blotting and analyzed using PY20 phosphotyrosine-specific monoclonal antibodies (Transduction Laboratories) or receptor-specific antiserum and the ECL detection method (Amersham Corp.).

The results of the experiment are presented in Figs. 14A and 14B. As shown in Fig. 14A, PAE cells expressing VEGFR-2 were stimulated with 10- or 2-fold concentrated medium from mock-transfected 293-EBNA cells (lanes 1 and 2), or with 2-, 5- or 10-fold concentrated medium from 293-EBNA cell cultures expressing the recombinant VEGF-C (lanes 3-6). VEGFR-2 was immunoprecipitated with specific antibodies and analyzed by SDS-PAGE and Western blotting using phosphotyrosine antibodies. For comparison, the stimulations were carried out with non-conditioned medium containing 50 ng/ml of purified recombinant VEGF (lanes 7 and 8). Lanes 6 and 7 show stimulation with VEGF-C- or VEGF-containing media pretreated with Flt4EC. As depicted in Fig. 14B, PDGFR-β-expressing NIH 3T3 cells were stimulated with non-conditioned medium (lane 1), 5-fold concentrated CM from mock-transfected (lane 2) or VEGF-C

- transfected (lanes 3 and 4) cells, or with non-conditioned medium containing 50 ng/ml of recombinant human PDGF-BB (lane 5). Medium containing VEGF-C was also pretreated with recombinant Flt4EC (lane 4). PDGFR-β was immunoprecipitated with specific antibodies and analyzed by SDS-PAGE and Western blotting using phosphotyrosine antibodies with subsequent stripping and reprobing of the membrane with antibodies specific for PDGFR-β.

Referring again to Fig. 14A, a basal level of tyrosine phosphorylation of VEGFR-2 was detected in cells stimulated by CM from the mock-transfected cells. A further concentration of this medium resulted in only a slight enhancement of VEGFR-2 phosphorylation (lanes 1 and 2). CM containing recombinant VEGF-C stimulated tyrosine autophosphorylation of VEGFR-2 and the intensity of the autophosphorylated polypeptide band was increased upon concentration of the VEGF-C CM (lanes 3-5). Furthermore, the stimulating effect was abolished after pretreatment of the medium with the Flt4EC affinity matrix (compare lanes 1, 5 and 6). The maximal effect of VEGF-C in this assay was comparable to the effect of recombinant VEGF added to unconditioned medium at concentration of 50 ng/ml (lane 8). Pretreatment of the medium containing VEGF with Flt4EC did not abolish its stimulating effect on VEGFR-2 (compare lanes 7 and 8). These results suggest that the VEGF-C expression vector encodes a ligand not only for Flt4 (VEGFR-3), but also for VEGFR-2 (Kdr).

In order to further confirm that the stimulating effect of VEGF-C on tyrosine phosphorylation of VEGFR-3 and VEGFR-2 was receptor-specific, we analyzed the effect of VEGF-C on tyrosine phosphorylation of PDGF receptor β (PDGFR-β) which is abundantly expressed on fibroblastic cells. As can be seen from Fig. 14B, a weak tyrosine phosphorylation of PDGFR-β was detected upon stimulation of Flt4-expressing NIH 3T3 cells with CM from

the mock-transfected cells (compare lanes 1 and 2). A similar low level of PDGFR-β phosphorylation was observed when the cells were incubated with CM from the VEGF-C transfected cells, with or without prior treatment with Flt4EC (lanes 3 and 4). In contrast, the addition of 50 ng/ml of PDGF-BB induced a prominent tyrosine autophosphorylation of PDGFR-β (lane 5).

EXAMPLE 15

VEGF-C Stimulates Endothelial Cell Migration In Collagen Gel

Conditioned media (CM) from cell cultures transfected with the VEGF-C expression vector was placed in a well made in collagen gel and used to stimulate the migration of bovine capillary endothelial (BCE) cells in the three-dimensional collagen gel as follows.

BCE cells (Folkman *et al., Proc. Natl. Acad. Sci. (USA), 76*:5217-5221 (1979) were cultured as described in Pertovaara *et al., J. Biol. Chem., 269*:6271-74 (1994). The collagen gels were prepared by mixing type I collagen stock solution (5 mg/ml in 1 mM HCl) with an equal volume of 2x MEM and 2 volumes of MEM containing 10% newborn calf serum to give a final collagen concentration of 1.25 mg/ml. The tissue culture plates (5 cm diameter) were coated with about 1 mm thick layer of the solution, which was allowed to polymerize at 37°C. BCE cells were seeded on top of this layer. For the migration assays, the cells were allowed to attach inside a plastic ring (1 cm diameter) placed on top of the first collagen layer. After 30 minutes, the ring was removed and unattached cells were rinsed away. A second layer of collagen and a layer of growth medium (5% newborn calf serum (NCS)), solidified by 0.75% low melting point agar (FMC BioProducts, Rockland, ME), were added. A well (3 mm diameter) was punched through all the layers on both sides of the cell spot at a distance of 4 mm, and the sample or control media were pipetted daily into the

wells. Photomicrographs of the cells migrating out from the spot edge were taken after six days through an Olympus CK 2 inverted microscope equipped with phase-contrast optics. The migrating cells were counted after nuclear staining with the fluorescent dye bisbenzimide (1 mg/ml, Hoechst 33258, Sigma).

Fig. 15 depicts a comparison of the number of cells migrating at different distances from the original area of attachment towards wells containing media conditioned by the non-transfected (control) or transfected (mock; VEGF-C; VEGF) cells, 6 days after addition of the media. The number of cells migrating out from the original ring of attachment was counted in five adjacent 0.5 mm x 0.5 mm squares using a microscope ocular lens grid and 10x magnification with a fluorescence microscope. Cells migrating further than 0.5 mm were counted in a similar way by moving the grid in 0.5 mm steps. The experiments were carried out twice with similar results, and medium values from the one of the experiments are presented with standard error bars. As can be seen from the columns, VEGF-C-containing CM stimulated cell migration more than medium conditioned by the non-transfected or mock-transfected cells but less than medium from cells transfected with a VEGF expression vector. Daily addition of 1 ng of FGF2 into the wells resulted in the migration of approximately twice the number of cells when compared to the stimulation by CM from VEGF-transfected cells.

EXAMPLE 16

VEGF-C Is Expressed In Multiple Tissues

Northern blots containing 2 micrograms of isolated poly(A)$^+$ RNA from multiple human tissues (blot from Clontech Laboratories, Inc., Palo Alto, CA) were probed with radioactively labelled insert of the 2.1 kb VEGF-C cDNA clone. Northern blotting and hybridization analysis showed that the 2.4 kb RNA and smaller amounts

of a 2.0 kb mRNA are expressed in multiple human tissues, most prominently in the heart, placenta, muscle, ovary and small intestine (Fig. 16A). Very little VEGF-C RNA was seen in the brain, liver or thymus and peripheral blood leukocytes (PBL) appeared negative. A similar analysis of RNA from human fetal tissues (Fig. 16B) shows that VEGF-C is highly expressed in the kidney and lung and to a lesser degree in the liver, while essentially no expression is detected in the brain. Interestingly, VEGF expression correlates with VEGF-C expression in these tissues, whereas VEGF-B is highly expressed in all tissues analyzed.

EXAMPLE 17

The VEGF-C Gene Localizes To Chromosome 4q34

A DNA panel of 24 interspecies somatic cell hybrids, which had retained one or two human chromosomes, was used for the chromosomal localization of the VEGF-C gene (Bios Laboratories, Inc., New Haven, CT). Primers were designed to amplify an about 250 bp fragment of the VEGF-C gene from somatic cell hybrid DNA. The primers and conditions for polymerase chain reaction (PCR) were 5'-TGAGTGATTTGTAGCTGCTGTG-3' (forward) [SEQ ID NO: 34] and 5'-TATTGCAGCAACCCCCACATCT-3' (reverse) [SEQ ID NO: 35] for VEGF-C (94°C, 60s/62°C, 45s/72°C, 60s). The PCR products were evaluated by electrophoresis in 1% agarose gels and visualized by ethidium bromide staining in ultraviolet light. [α-^{32}P]-dCTP- labelled cDNA inserts of a plasmid representing the complete VEGF-C coding domain was used as a probe in Southern blotting and hybridization analysis of the somatic cell hybrid DNAs as instructed by the supplier (Bios Laboratories).

The cell lines for fluorescence *in situ* hybridization (FISH) were obtained from the American Type Culture Collection (Rockville, MD). Purified DNA from P1 clones 7660 and 7661 (VEGF-C) (Genome Systems, Inc., St. Louis, MO) were confirmed positive by Southern blotting

of *Eco*RI- digested DNA followed by hybridization with the VEGF-C cDNA. The P1 clones were then labelled by nick translation either with biotin-11-dUTP, biotin-14-ATP (Sigma Chemical Co., St. Louis, MO) or digoxigenin 11-dUTP (Boehringer Mannheim GmbH, Mannheim, Germany) according to standard protocols. PHA-stimulated peripheral blood lymphocyte cultures were treated with 5-bromodeoxyuridine (BrdU) at an early replicating phase to induce G-banding. See Takahashi *et al.*, *Human Genet.*, *86:*14-16 (1995); Lemieux *et al.*, *Cytogenet. Cell Genet.*, *59:*311-12 (1992). The FISH procedure was carried out in 50% formamide, 10% dextran sulphate in 2x SSC using well-known procedures. *See e.g.*, Rytkönnen *et al.*, *Cytogenet Cell Genet.*, *68:*61-63 (1995); Lichter *et al.*, *Proc. Natl. Acad. Sci. (USA)*, *85:*9664-68 (1988). Repetitive sequences were suppressed with 50-fold excess of Cot-1 DNA (BRL, Gaithersburg, MD) compared with the labeled probe. Specific hybridization signals were detected by incubating the hybridized slides in labelled antidigoxigenin antibodies, followed by counterstaining with 0.1mmol/L 4,6-diamino-2-phenylindole. Probe detection for two-color experiments was accomplished by incubating the slides in fluorescein isothiocyanate (FITC)-anti-digoxigenin antibodies (Sigma Chemical Co.) and Texas red-avidin (Vector Laboratories, Burlingame, CA) or rhodamine-anti-digoxigenin and FITC-avidin.

Multi-color digital image analysis was used for acquisition, display and quantification of hybridization signals of metaphase chromosomes. The system contains a PXL camera (Photometrics Inc., Tucson, AZ) attached to a PowerMac 7100/Av workstation. IPLab software controls the camera operation, image acquisition and Ludl Filter wheel. At least 50 nuclei were scored. Overlapping nuclei and clusters of cells were ignored. A slide containing normal lymphocyte metaphase spreads and interphase nuclei was included in each experiment to control for the efficiency and specificity of the

hybridization.

In order to determine the chromosomal localization of the human VEGF-C gene, DNAs from human rodent somatic cell hybrids containing defined sets of human chromosomes were analyzed by Southern blotting and hybridization with the VEGF-C cDNA probe. Among 24 DNA samples on the hybrid panel, representing different human chromosomes, human-specific signals were observed only in hybrids which contained human chromosome 4. The results were confirmed by PCR of somatic cell hybrid DNAs using VEGF-C specific primers, where amplified bands were obtained only from DNAs containing human chromosome 4.

A genomic P1 plasmid for VEGF-C was isolated using specific primers and PCR and verified by Southern blotting and hybridization using a VEGF-C specific cDNA probe. The chromosomal localization of VEGF-C was further studied using metaphase FISH. Using the P1 probe for VEGF-C in FISH a specific hybridization to the 4q34 chromosomal band was detected in 40 out of 44 metaphases. Double-fluorochrome hybridization using a cosmid probe specific for the aspartylglucosaminidase (AGA) gene showed that VEGF-C is located just proximal to the AGA gene previously mapped to the 4q34-35 chromosomal band.

Biotin labelled VEGF-C P1 and digoxigenin labeled AGA cosmid probes were hybridized simultaneously to metaphase chromosomes. This experiment demonstrated that the AGA gene is more telomerically located than the VEGF-C gene. The foregoing example demonstrates the utility of polynucleotides of the invention as chromosomal markers and for the presence or absence of the VEGF-C gene region in normal or diseased cells. The VEGF-C locus at 4q34 is a candidate target for mutations leading to vascular malformations or cardiovascular diseases.

EXAMPLE 18

Effect of glucose concentration and hypoxia on VEGF, VEGF-B and VEGF-C mRNA levels in C6 glioblastoma cells

Confluent cultures of C6 cells (ATCC CCL 107) were grown on 10 cm diameter tissue culture plates containing 2.5 ml of DMEM and 5% fetal calf serum plus antibiotics. The cultures were exposed for 16 hours to normoxia in a normal cell culture incubator containing 5% CO_2 or hypoxia by closing the culture plates in an airtight glass chamber and burning a piece of wood inside until the flame was extinguished due to lack of oxygen. Polyadenylated RNA was isolated (as in the other examples), and 8 micrograms of the RNA was electrophoresed and blot-hybridized with a mixture of the VEGF, VEGF-B and VEGF-C probes (see Fig. 12). The results show that hypoxia strongly induces VEGF mRNA expression, both in low and high glucose, but has no significant effect on the VEGF-B mRNA levels. The VEGF-C mRNA isolated from hypoxic cells runs slightly faster in gel electrophoresis and an extra band of faster mobility can be seen below the upper mRNA band. This observation suggests that hypoxia affects VEGF-C RNA processing. One explanation for this observation is that VEGF-C mRNA splicing is altered, affecting the VEGF-C open reading frame and resulting in an alternative VEGF-C protein being produced by hypoxic cells. Such alternative forms of VEGF-C and VEGF-C-encoding polynucleotides are contemplated as an aspect of the invention. This data indicates screening and diagnostic utilities for polynucleotides and polypeptides of the invention, such as methods whereby a biological sample is screened for the hypoxia-induced form of VEGF-C and/or VEGF-C mRNA. The data further suggests a therapeutic indication for antibodies and/or other inhibitors of the hypoxia-induced form of VEGF-C or the normal form of VEGF-C.

EXAMPLE 19

Pulse-chase labeling and immunoprecipitation of VEGF-C polypeptides from 293 EBNA cells transfected with VEGF-C expression vector.

The following VEGF-C branched amino-terminal peptide, designated PAM126, was synthesized for production of anti-VEGF-C antiserum:

NH_2-E-E-T-I-K-F-A-A-A-H-Y-N-T-E-I-L-K-COOH (SEQ ID NO: 39).

In particular, PAM126 was synthesized as a branched polylysine structure K3PA4 having four peptide acid (PA) chains attached to two available lysine (K) residues. The synthesis was performed on a 433A Peptide Synthesizer (Applied Biosystems) using Fmoc-chemistry and TentaGel S MAP RAM10 resin mix (RAPP Polymere GmbH, Tubingen, Germany), yielding both cleavable and resin-bound peptides. The cleavable peptide was purified via reverse phase HPLC and was used together with the resin-bound peptide in immunizations. The correctness of the synthesis products were confirmed using mass-spectroscopy (Lasermatt).

The PAM126 peptide was dissolved in phosphate buffered saline (PBS), mixed with Freund's adjuvant, and used for immunization of rabbits at bi-weekly intervals using methods standard in the art (Harlow and Lane, *Antibodies, a laboratory manual*, Cold Spring Harbor Laboratory Press (1988)). Antisera obtained after the fourth booster immunization was used for immunoprecipitation of VEGF-C in pulse-chase experiments, as described below.

For pulse-chase analysis, 293 EBNA cells transfected with a VEGF-C expression vector (i.e., the FLT4-L cDNA inserted into the pREP7 expression vector as described above) were incubated for 30 minutes in methionine-free, cysteine-free, serum-free DMEM culture medium at 37°C. The medium was then changed, and 200 μCi of Pro-mix™ (Amersham), was added. The cell layers were incubated in this labeling medium for two hours, washed

with PBS, and incubated for 0, 15, 30, 60, 90, 120, or 180 minutes in serum-free DMEM (chase). After the various chase periods, the medium was collected, the cells were again washed two times in PBS, and lysed in immunoprecipitation buffer. The VEGF-C polypeptides were analyzed from both the culture medium and from the cell lysates by immunoprecipitation, using the VEGF-C-specific antiserum raised against the NH_2-terminal peptide (PAM126) of the 23 kD VEGF-C form. Immunoprecipitated polypeptides were analyzed via SDS-PAGE followed by autoradiography.

Referring to Fig. 19, the resultant autoradiograms demonstrate that immediately after a 2 hour labeling (chase time 0), the VEGF-C vector-transfected cells contained a radioactive 55 kD polypeptide band, which is not seen in mock-transfected cells (M). This 55 kD polypeptide band gradually diminishes in intensity with increasing chase periods, and is no longer detected in the cells by 180 minutes of chase. A 32 kD polypeptide band also is observed in VEGF-C transfected cells (and not mock-transfected cells). This 32 kD band disappears with similar kinetics to that of the 55 kD band. Simultaneously, increasing amounts of 32 kD (arrow) and subsequently 23 kD (arrow) and 14 kD polypeptides appear in the medium.

Collectively, the data from the pulse-chase experiments indicate that the 55 kD intracellular polypeptide represents a pro-VEGF-C polypeptide, which is not secreted from cells, but rather is first proteolytically cleaved into the 32 kD form. The 32 kD form is secreted and simultaneously further processed by proteolysis into the 23 kD and 14 kD forms. Without intending to be limited to a particular theory, it is believed that processing of the VEGF-C precursor occurs as removal of a signal sequence, removal of the COOH-terminal domain (BR3P), and removal of an amino terminal polypeptide, resulting in a VEGF-C polypeptide

having the TEE... amino terminus.

At high resolution, the 23 kD polypeptide band appears as a closely-spaced polypeptide doublet, suggesting heterogeneity in cleavage or glycosylation.

EXAMPLE 20

Isolation of Mouse and Quail cDNA Clones Encoding VEGF-C

To clone a mouse variant of VEGF-C, approximately 1 x 10^6 bacteriophage lambda clones of a commercially-available 12 day mouse embryonal cDNA library (lambda EXlox library, Novagen, catalog number 69632-1) were screened with a radiolabeled fragment of human VEGF-C cDNA containing nucleotides 495 to 1661 of SEQ ID NO: 32. One positive clone was isolated.

A 1323 bp *Eco*RI/*Hin*dIII fragment of the insert of the isolated mouse cDNA clone was subcloned into the corresponding sites of the pBluescript SK+ vector (Stratagene) and sequenced. The cDNA sequence of this clone was homologous to the human VEGF-C sequence reported herein, except that about 710 bp of 5'-end sequence present in the human clone was not present in the mouse clone.

For further screening of mouse cDNA libraries, a *Hin*dIII-*Bst*XI (*Hin*dIII site is from the pBluescript SK+ polylinker) fragment of 881 bp from the coding region of the mouse cDNA clone was radiolabeled and used as a probe to screen two additional mouse cDNA libraries. Two additional cDNA clones from an adult mouse heart ZAP II cDNA library (Stratagene, catalog number 936306) were identified. Three additional clones also were isolated from a mouse heart 5'-stretch-plus cDNA library in λgt11 (Clontech Laboratories, Inc., catalog number ML5002b). Of the latter three clones, one was found to contain an insert of about 1.9 kb. The insert of this cDNA clone was subcloned into *Eco*RI sites of pBluescript SK+ vector and both strands of this clone were completely sequenced, resulting in the nucleotide and deduced amino acid

sequences shown in SEQ ID NOs: 40 and 41.

It is contemplated that the polypeptide corresponding to SEQ ID NO: 41 is processed into a mature mouse VEGF-C protein, in a manner analogous to the processing of the human VEGF-C prepropeptide. Putative cleavage sites for the mouse protein are identified using procedures outlined above for identification of cleavage sites for the human VEGF-C polypeptide.

The foregoing results demonstrate the utility of polynucleotides of the invention for identifying and isolating polynucleotides encoding other non-human mammalian variants of VEGF-C. Such identified and isolated polynucleotides, in turn, can be expressed (using procedures similar to those described in preceding examples) to produce recombinant polypeptides corresponding to non-human mammalian variants of VEGF-C.

The mouse and human VEGF-C sequences were used to design probes for isolating a quail VEGF-C cDNA from a quail cDNA library. A fragment of the human VEGF-C cDNA comprising nucleotides 495-1670 of SEQ ID NO: 32 was obtained by PCR amplification, cloned into the pCRII vector (Invitrogen) according to the manufacturer's instructions, and amplified. The insert was isolated by *Eco* RI digestion and preparative gel electrophoresis and then labelled using radioactive dCTP and random priming. A cDNA library made from quail embryos of stage E-4 in pcDNA-1 vector (Invitrogen) was then screened using this probe. About 200,000 colonies were plated and filter replicas were hybridized with the radioactive probe. Nine positive clones were identified and secondarily plated. Two of the nine clones hybridized in secondary screening. The purified clones (clones 1 and 14) had approximately 2.7 kb *Eco* RI inserts. Both clones were amplified and then sequenced using the T7 and SP6 primers (annealing to the vector). In addition, an internal *Sph* I restriction endonuclease cleavage site was identified

about 1.9 kb from the T7 primer side of the vector and used for subcloning 5′- and 3′- *Sph* I fragments, followed by sequencing from the *Sph* I end of the subclones. The sequences obtained were identical from both clones and showed a high degree of similarity to the human VEGF-C coding region. Subsequently, walking primers were made in both directions and double-stranded sequencing was completed for 1743 base pairs, including the full-length open reading frame.

The cDNA sequence obtained includes a long open reading frame and 5′ untranslated region. The DNA and deduced amino acid sequences for the quail cDNA are set forth in SEQ ID NOs: 52 and 53, respectively. As shown in Fig. 8, the human, murine, and avian (quail) VEGF-C precursor amino acid sequences share a significant degree of conservation. This high degree of homology permits the isolation of VEGF-C encoding sequences from other species, especially vertebrate species, and more particularly mammalian and avian species, using polynucleotides of the present invention as probes and using standard molecular biological techniques such as those described herein.

EXAMPLE 21

N-terminal peptide sequence analyses of recombinant VEGF-C

Cells (293 EBNA) transfected with VEGF-C cDNA (see Example 13) secrete several forms of recombinant VEGF-C (Fig. 21A, lane IP). In the absence of alkylation, the three major, proteolytically-processed forms of VEGF-C migrate in SDS-PAGE as proteins with apparent molecular masses of 32/29 kD (doublet), 21 kD and 15 kD. Two minor polypeptides exhibit approximate molecular masses of 63 and 52 kD, respectively. One of these polypeptides is presumably a glycosylated and non-processed form; the other polypeptide is presumably glycosylated and partially processed.

To determine sites of proteolytic cleavage of

the VEGF-C precursor, an immunoaffinity column was used to purify VEGF-C polypeptides from the conditioned medium of 293 EBNA cells transfected with VEGF-C cDNA. To prepare the immunoaffinity column, a rabbit was immunized with a synthetic peptide corresponding to amino acids 104-120 of SEQ ID NO: 33: H_2N-EETIKFAAAHYNTEILK (see PAM126 in Example 19). The IgG fraction was isolated from the serum of the immunized rabbit using protein A Sepharose (Pharmacia). The isolated IgG fraction was covalently bound to CNBr-activated Sepharose CL-4B (Pharmacia) using standard techniques at a concentration of 5 mg IgG/ml of Sepharose. This immunoaffinity matrix was used to isolate processed VEGF-C from 1.2 liters of the conditioned medium (CM).

The purified material eluted from the column was analyzed by gel electrophoresis and Western blotting. Fractions containing VEGF-C polypeptides were combined, dialyzed against 10 mM Tris HCl, vacuum-dried, electrotransferred to Immobilon-P (polyvinylidene difluoride or PVDF) transfer membrane (Millipore, Marlborough, MA) and subjected to N-terminal amino acid sequence analysis.

The polypeptide band of 32 kD yielded two distinct sequences: NH_2-FESGLDLSDA... and NH_2-AVVMTQTPAS... (SEQ ID NO: 51), the former corresponding to the N-terminal part of VEGF-C after cleavage of the signal peptide, starting from amino acid 32 (SEQ ID NO: 33), and the latter corresponding to the kappa-chain of IgG, which was present in the purified material due to "leakage" of the affinity matrix during the elution procedure.

In order to obtain the N-terminal peptide sequence of the 29 kD form of VEGF-C, a construct (VEGF-C NHis) encoding a VEGF-C variant was generated. In particular, the construct encoded a VEGF-C variant that fused a 6xHis tag to the N-terminus of the secreted precursor (*i.e.*, between amino acids 31 and 33 in SEQ ID

NO: 33). The phenylalanine at position 32 was removed to prevent possible cleavage of the tag sequence during secretion of VEGF-C. The VEGF-C NHis construct was cloned into pREP7 as a vector; the construction is described more fully in Example 28, below.

The calcium phosphate co-precipitation technique was used to transfect VEGF-C NHis into 293 EBNA cells. Cells were incubated in DMEM/10% fetal calf serum in 15 cm cell culture dishes (a total of 25 plates). The following day, the cells were reseeded into fresh culture dishes (75 plates) containing the same medium and incubated for 48 hours. Cell layers were then washed once with PBS and DMEM medium lacking FCS was added. Cells were incubated in this medium for 48 hours and the medium was collected, cleared by centrifugation at 5000 x *g* and concentrated 500X using an Ultrasette Tangential Flow Device (Filtron, Northborough, MA), as described in Example 5 above. VEGF-C NHis was purified from the concentrated conditioned medium using TALON™ Metal Affinity Resin (Clontech Laboratories, Inc.) and the manufacturer's protocol for native protein purification using imidazole-containing buffers. The protein was eluted with a solution containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 200 mM imidazole. The eluted fractions containing purified VEGF-C NHis were detected by immunoblotting with Antiserum 882 (antiserum from rabbit 882, immunized with the PAM-126 polypeptide). Fractions containing VEGF-C NHis were combined, dialyzed and vacuum-dried. As can be seen in Fig. 27, due to the presence of the 6xHis tag at the N-terminus of this form of VEGF-C, the upper component of the major doublet of the VEGF-CNHis migrates slightly slower than the 32 kD form of wild type VEGF-C, thereby improving the separation of the VEGF-CNHis 32 kD variant from the 29 kD band using SDS-PAGE. Approximately 15 μg of the purified VEGF-C were subjected to SDS-PAGE under reducing conditions, electrotransferred to Immobilon-P (PVDF)

transfer membrane (Millipore, Inc., Marlborough, MA) and the band at 29 kD was subjected to N-terminal amino acid sequence analysis. This sequence analysis revealed an N-terminal sequence of H_2N-SLPAT . . ., corresponding to amino acids 228-232 of VEGF-C (SEQ ID NO: 33).

The polypeptide band of 21 kD yielded the sequence H_2N-AHYNTEILKS . . ., corresponding to an amino-terminus starting at amino acid 112 of SEQ ID NO: 33. Thus, the proteolytic processing site which results in the 21 kD form of VEGF-C produced by transfected 293 EBNA cells apparently occurs nine amino acid residues downstream of the cleavage site which results in the 23 kD form of VEGF-C secreted by PC-3 cells.

The N-terminus of the 15 kD form was identical to the N-terminus of the 32 kD form (NH_2-FESGLDLSDA...). The 15 kD form was not detected when recombinant VEGF-C was produced by COS cells. This suggests that production of this form is cell lineage specific.

Example 22

Dimeric and monomeric forms of VEGF-C

The composition of VEGF-C dimers was analyzed as follows. Cells (293 EBNA cells), transfected with the pREP7 VEGF-C vector as described in Example 11, were metabolically labelled with Pro-mix L-[^{35}S] labelling mix (Amersham Corp.) to a final concentration of 100 μCi/ml.

In parallel, a VEGF-C mutant, designated "R102S", was prepared and analyzed. To prepare the DNA encoding VEGF-C-R102S, the arginine codon at position 102 of SEQ ID NO: 33 was replaced with a serine codon. This VEGF-C-R102S-encoding DNA, in a pREP7 vector, was transfected into 293 EBNA cells and expressed as described above. VEGF-C polypeptides were immunoprecipitated using antisera 882 (obtained by immunization of a rabbit with a polypeptide corresponding to residues 104-120 of SEQ ID NO: 33 (see previous Example)) and antisera 905 (obtained by immunization of a

rabbit with a polypeptide corresponding to a portion of the prepro- VEGF-C leader: H_2N-ESGLDLSDAEPDAGEATAYASK (residues 33 to 54 of SEQ ID NO: 33).

The immunoprecipitates from each cell culture were subjected to SDS-PAGE under non-denaturing conditions (Fig. 21B). Bands 1-6 were cut out from the gel, soaked for 30 minutes in 1x gel-loading buffer containing 200 mM ß-mercaptoethanol, and individually subjected to SDS-PAGE under denaturing conditions (Figs. 21A and 21C, lanes 1-6).

As can be seen from Figures 21A-C, each high molecular weight form of VEGF-C (Fig. 21B, bands 1-4) consists of at least two monomers bound by disulfide bonds (Compare Figs. 21A and 21C, lanes 1-4, in the reducing gels). The main component of bands 1-3 is the doublet of 32/29 kD, where both proteins are present in an equimolar ratio. The main fraction of the 21 kD form is secreted as either a monomer or as a homodimer connected by means other than disulfide bonds (bands 6 and lanes 6 in Figs. 21A-C).

The R102S mutation creates an additional site for N-linked glycosylation in VEGF-C at the asparagine residue at position 100 in SEQ ID NO: 33. Glycosylation at this additional glycosylation site increases the apparent molecular weight of polypeptides containing the site, as confirmed in Figures 21A-C and Figures 22A-B. The additional glycosylation lowers the mobility of forms of VEGF-C-R102S that contain the additional glycosylation site, when compared to polypeptides of similar primary structure corresponding to VEGF-C. Figures 21A-C and Figures 22A-B reveal that the VEGF-C-R102S polypeptides corresponding to the 32 kD and 15 kD forms of wt VEGF-C exhibit increased apparent molecular weights, indicating that each of these polypeptides contains the newly introduced glycosylation site. In particular, the VEGF-C-R102S polypeptide corresponding to the 15 kD polypeptide from VEGF-C comigrates on a gel with the 21

kD form of the wild type (wt) VEGF-C, reflecting a shift on the gel to a position corresponding to a greater apparent molecular weight. (Compare lanes 4 in Figures 21A and 21C).

In a related experiment, another VEGF-C mutant, designated "R226,227S," was prepared and analyzed. To prepare a DNA encoding VEGF-C-R226,227S, the arginine codons at positions 226 and 227 of SEQ ID NO: 33 were replaced with serine codons by site-directed mutagenesis. The resultant DNA was transfected into 293 EBNA cells as described above and expressed and analyzed under the same conditions as described for VEGF-C and VEGF-C-R102S. In the conditioned medium from the cells expressing VEGF-C-R226,227S, no 32 kD form of VEGF-C was detected. These results indicate that a C-terminal cleavage site of wild-type VEGF-C is adjacent to residues 226 and 227 of SEQ ID NO: 33, and is destroyed by the mutation of the arginines to serines. Again, the mobility of the 29 kD component of the doublet was unchanged (Figures 22A-B).

Taken together, these data indicate that the major form of the processed VEGF-C is a heterodimer consisting of (1) a polypeptide of 32 kD containing amino acids 32-227 of the prepro-VEGF-C (amino acids 32 to 227 in SEQ ID NO: 33) attached by disulfide bonds to (2) a polypeptide of 29 kD beginning with amino acid 228 in SEQ ID NO: 33. These data are also supported by a comparison of the pattern of immunoprecipitated, labelled VEGF-C forms using antisera 882 and antisera 905.

When VEGF-C immunoprecipitation was carried out using conditioned medium, both antisera (882 and 905) recognized some or all of the three major processed forms of VEGF-C (32/29 kD, 21 kD and 15 kD). When the conditioned medium was reduced by incubation in the presence of 10 mM dithiothreitol for two hours at room temperature with subsequent alkylation by additional incubation with 25 mM iodoacetamide for 20 minutes at room temperature, neither antibody precipitated the 29 kD

component, although antibody 882 still recognized polypeptides of 32 kD, 21 kD and 15 kD. These results are consistent with the nature of the oligopeptide antigen used to elicit the antibodies contained in antisera 882, an oligopeptide containing amino acid residues 104-120 of SEQ ID NO: 33. On the other hand, antisera 905 recognized only the 32 kD and 15 kD polypeptides, which include sequence of the oligopeptide (amino acids 33 to 54 of SEQ ID NO: 33) used for immunization to obtain antisera 905. Taking into account the mobility shift of the 32 kD and 15 kD forms, the immunoprecipitation results with the R102S mutant were similar (Figs. 23A-B). The specificity of antibody 905 is confirmed by the fact that it did not recognize a VEGF-C ΔN variant form wherein the N-terminal propeptide spanning residues 32-102 of the unprocessed polypeptide had been deleted (Fig. 23B).

The results of these experiments also demonstrate that the 21 kD polypeptide is found (1) in heterodimers with other molecular forms (see Figs. 21A-C and Figs. 22A-B), and (2) secreted as a monomer or a homodimer held by bonds other than disulfide bonds (Figs. 21A and 21B, lanes 6).

The experiments disclosed in this example demonstrate that several forms of VEGF-C exist. A variety of VEGF-C monomers were observed and these monomers can vary depending on the level and pattern of glycosylation. In addition, VEGF-C was observed as a multimer, for example a homodimer or a heterodimer. The processing of VEGF-C is schematically presented in Fig. 18 (disulfide bonds not shown). All forms of VEGF-C are within the scope of the present invention.

Example 23

***In situ* Hybridization of Mouse Embryos**

To analyze VEGF-C mRNA distribution in different cells and tissues, sections of 12.5 and 14.5-

day post-coitus (p.c.) mouse embryos were prepared and analyzed via *in situ* hybridization using labeled VEGF-C probes. *In situ* hybridization of tissue sections was performed as described in Västrik *et al.*, *J. Cell Biol.*, *128*:1197-1208 (1995). A mouse VEGF-C antisense RNA probe was generated from linearized pBluescript II SK+ plasmid (Stratagene Inc., La Jolla, CA), containing a cDNA fragment corresponding to nucleotides 499-979 of a mouse VEGF-C cDNA (SEQ ID NO: 40). Radiolabeled RNA was synthesized using T7 polymerase and [^{35}S]-UTP (Amersham). Mouse VEGF-B antisense and sense RNA probes were synthesized in a similar manner from linearized pCRII plasmid containing the mouse VEGF-B cDNA insert as described Olofsson *et al.*, *Proc. Natl. Acad. Sci. (USA)*, *93*:2576-2581 (1996). The high stringency wash was for 45 minutes at 65°C in a solution containing 30 mM dithiothreitol (DTT) and 4 x SSC. The slides were exposed for 28 days, developed and stained with hematoxylin. For comparison, similar sections were hybridized with a VEGFR-3 probe and the 12.5-day p.c. embryos were also probed for VEGF-B mRNA.

Figures 34A-D show darkfield (Figures 34A-C) and lightfield (Figure 34D) photomicrographs of 12.5 day p.c. embryo sections probed with the antisense (Fig. 34A) and sense (Figs. 34C-D) VEGF-C probes. Fig. 34A illustrates a parasagittal section, where VEGF-C mRNA is particularly prominent in the mesenchyme around the vessels surrounding the developing metanephros (mn). In addition, hybridization signals were observed between the developing vertebrae (vc), in the developing lung mesenchyme (lu), in the neck region and developing forehead. The specificity of these signals is evident from the comparison with VEGF-B expression in an adjacent section (Fig. 34B), where the myocardium gives a very strong signal and lower levels of VEGF-B mRNA are detected in several other tissues. Both genes appear to be expressed in between the developing vertebrae (vc), in

the developing lung (lu) and forehead. Hybridization of the VEGF-C sense probe showed no specific expression within these structures (Fig. 34C).

Figs. 35A-D show a comparison of the expression patterns of VEGF-C and VEGFR-3 in 12.5 day p.c. mouse embryos in the jugular region, where the developing dorsal aorta and cardinal vein are located. This is the area where the first lymphatic vessels sprout from venous sac-like structures according to the long-standing theory of Sabin, *Am. J. Anat.*, *9*:43-91 (1909). As can be seen from Figs. 35A-D, an intense VEGF-C signal is detected in the mesenchyme surrounding the developing venous sacs (Figs. 35A and 35C) which are positive for VEGFR-3 (Figs. 35B and 35D).

The mesenterium supplies the developing gut with blood and contains developing lymphatic vessels. The developing 14.5 day p.c. mesenterium is positive for VEGF-C mRNA, with particularly high expression in connective tissue surrounding certain vessels (arrowheads in Figs. 35E-H). This signal in Fig. 35E should be distinguished from the false positive reflection of light from red blood cells within the vessel. The adjacent mesenterial VEGFR-3 signals shown in Fig. 35F originate from small capillaries of the mesenterium (arrowhead). Therefore, there appears to be a paracrine relationship between the production of the mRNAs for VEGF-C and its receptor. This data indicates that VEGF-C is expressed in a variety of tissues. Moreover, the pattern of expression is consistent with a role for VEGF-C in venous and lymphatic vessel development. Further, the data reveals that VEGF-C is expressed in non-human animals.

Example 24

Analysis of VEGF, VEGF-B, and VEGF-C mRNA Expression in Fetal and Adult Tissues

A human fetal tissue Northern blot containing 2 μg of polyadenylated RNAs from brain, lung, liver and kidney (Clontech Inc.) was hybridized with a pool of the

following probes: a human full-length VEGF-C cDNA insert (Genbank Acc. No. X94216), a human VEGF-B_{167} cDNA fragment (nucleotides 1-382, Genbank Acc. No. U48800) obtained by PCR amplification; and a human VEGF 581 bp cDNA fragment covering base pairs 57-638 (Genbank Acc. No. X15997). Blots were washed under stringent conditions, using techniques standard in the art.

Mouse embryo multiple tissue Northern blot (Clontech Inc.) containing 2 μg of polyadenylated RNAs from 7, 11, 15 and 17 day postcoital (p.c.) embryos was hybridized with mouse VEGF-C cDNA fragment (base pairs 499-656). A mouse adult tissue Northern blot was hybridized with the probes for human VEGF, VEGF-B_{167}, VEGF-C and with a VEGFR-3 cDNA fragment (nucleotides 1-595; Genbank Acc. No. X68203).

In adult mouse tissues, both 2.4 kb and 2.0 kb mRNA signals were observed with the VEGF-C probe, at an approximately 4:1 ratio. The most conspicuous signals were obtained from lung and heart RNA, while kidney, liver, brain, and skeletal muscle had lower levels, and spleen and testis had barely visible levels. As in the human tissues, VEGF mRNA expression in adult mice was most abundant in lung and heart RNA, whereas the other samples showed less coordinate regulation with VEGF-C expression. Skeletal muscle and heart tissues gave the highest VEGF-B mRNA levels from adult mice, as previously reported Olofsson *et al., Proc. Natl. Acad. Sci. (USA), 93*:2576-2581 (1996). Comparison with VEGFR-3 expression showed that the tissues where VEGF-C is expressed also contain mRNA for its cognate receptor tyrosine kinase, although in the adult liver VEGFR-3 mRNA was disproportionally abundant.

To provide a better insight into the regulation of the VEGF-C mRNA during embryonic development, polyadenylated RNA isolated from mouse embryos of various gestational ages (7, 11, 15, and 17 day p.c.) was hybridized with the mouse VEGF-C probe. These analyses

showed that the amount of 2.4 kb VEGF-C mRNA is relatively constant throughout the gestational period.

Example 25

Regulation of mRNAs for VEGF family members by serum, interleukin-1 and dexamethasone in human fibroblasts in culture

Human IMR-90 fibroblasts were grown in DMEM medium containing 10% FCS and antibiotics. The cells were grown to 80% confluence, then starved for 48 hours in 0.5 % FCS in DMEM. Thereafter, the growth medium was changed to DMEM containing 5% FCS, with or without 10 ng/ml interleukin-1 (IL-1) and with or without 1 mM dexamethasone, as indicated in Figs. 24A-B. The culture plates were incubated with these additions for the times indicated, and total cellular RNA was isolated using the TRIZOL kit (GIBCO-BRL). About 20 μg of total RNA from each sample was electrophoresed in 1.5% formaldehyde-agarose gels as described in Sambrook *et al., supra* (1989). The gel was used for Northern blotting and hybridization with radiolabeled insert DNA from the human VEGF clone (a 581 bp cDNA covering bps 57-638, Genbank Acc. No. 15997) and a human VEGF-B_{167} cDNA fragment (nucleotides 1-382, Genbank Acc. No. U48800) (Fig. 25B). Subsequently, the Northern blots were probed with radiolabelled insert from the VEGF-C cDNA plasmid (Fig. 24A). Primers were labelled using a standard technique involving enzymatic extension reactions of random primers, as would be understood by one of ordinary skill in the art. The mobilities of the 28S and 18S ribosomal RNA bands are indicated, based on UV photography of ethidium bromide stained RNA before the transfer.

As can be seen in Figs. 24A-B, very low levels of VEGF-C and VEGF are expressed by the starved IMR-90 cells as well as cells after 1 hour of stimulation. In contrast, abundant VEGF-B mRNA signal is visible under these conditions. After a 4 hours of serum stimulation, there is a strong induction of VEGF-C and VEGF mRNAs,

which are further increased in the IL-1 treated sample. The effect of IL-1 seems to be abolished in the presence of dexamethasone. A similar pattern of enhancement is maintained in the 8 hour sample, but a gradual down-regulation of all signals occurs for both RNAs in the 24 hour and 48 hour samples. In contrast, VEGF-B mRNA levels remain constant and thus show remarkable stability throughout the time period. The results are useful in guiding efforts to use VEGF-C and its fragments, its antagonists, and anti-VEGF-C antibodies in methods for treating a variety of disorders.

Example 26

Expression and analysis of recombinant murine VEGF-C

The mouse VEGF-C cDNA was expressed as a recombinant protein and the secreted protein was analyzed for its receptor binding properties. The binding of mouse VEGF-C to the human VEGFR-3 extracellular domain was studied by using media from Bosc23 cells transfected with mouse VEGF-C cDNA in a retroviral expression vector.

The 1.8 kb mouse VEGF-C cDNA was cloned as an *Eco*RI fragment into the retroviral expression vector pBabe-puro containing the SV40 early promoter region [Morgenstern *et al., Nucl. Acids Res., 18*:3587-3595 (1990)], and transfected into the Bosc23 packaging cell line [Pearet *et al., Proc. Natl. Acad. Sci. (USA), 90*:8392-8396 (1994)] by the calcium-phosphate precipitation method. For comparison, Bosc23 cells also were transfected with the previously- described human VEGF-C construct in the pREP7 expression vector. The transfected cells were cultured for 48 hours prior to metabolic labelling. Cells were changed into DMEM medium devoid of cysteine and methionine, and, after 45 minutes of preincubation and medium change, Pro-mix™ L-[^{35}S] *in vitro* cell labelling mix (Amersham Corp.), in the same medium, was added to a final concentration of about 120 μCi/ml. After 6 hours of incubation, the culture medium

was collected and clarified by centrifugation.

For immunoprecipitation, 1 ml aliquots of the media from metabolically-labelled Bosc23 cells transfected with empty vector or mouse or human recombinant VEGF-C, respectively, were incubated overnight on ice with 2 μl of rabbit polyclonal antiserum raised against an N-terminal 17 amino acid oligopeptide of mature human VEGF-C (H_2N-EETIKFAAAHYNTEILK) (SEQ ID NO: 33, residues 104-120). Thereafter, the samples were incubated with protein A sepharose for 40 minutes at 4°C with gentle agitation. The sepharose beads were then washed twice with immunoprecipitation buffer and four times with 20 mM Tris-HCl, pH 7.4. Samples were boiled in Laemmli buffer and analyzed by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoprecipitation of VEGF-C from media of transfected and metabolically-labelled cells revealed bands of approximately 30-32x10^3 M_r (a doublet) and 22-23x10^3 M_r in 12.5% SDS-PAGE. These bands were not detected in samples from nontransfected or mock-transfected cells as shown in Fig. 32 (*i.e.*, lanes marked "vector"). These results show that antibodies raised against human VEGF-C recognize the corresponding mouse ligand.

For receptor binding experiments, 1 ml aliquots of media from metabolically-labelled Bosc23 cells were incubated with VEGFR-3 extracellular domain (see Example 3), covalently coupled to sepharose, for 4 hours at 4°C with gentle mixing. The sepharose beads were washed four times with ice-cold phosphate buffered saline (PBS), and the samples were analyzed by gel electrophoresis as described in Joukov *et al.*, *EMBO J.*, *15*:290-298 (1996).

As can be seen from Fig. 32, similar 30-32 x 10^3 M_r doublet and 22-23 x 10^3 M_r polypeptide bands were obtained in the receptor binding assay as compared to the immunoprecipitation assay. Thus, mouse VEGF-C binds to human VEGFR-3. The slightly faster mobility of the mouse

VEGF-C polypeptides may be caused by the four amino acid residue difference observed in sequence analysis (residues H88-E91, Fig. 31).

The capacity of mouse recombinant VEGF-C to induce VEGFR-3 autophosphorylation was also investigated. For the VEGFR-3 receptor stimulation experiments, subconfluent NIH 3T3-Flt4 cells, Pajusola *et al., Oncogene, 9*:3545-3555 (1994), were starved overnight in serum-free medium containing 0.2% BSA. In general, the cells were stimulated with the conditioned medium from VEGF-C vector-transfected cells for 5 minutes, washed three times with cold PBS containing 200 μM vanadate, and lysed in RIPA buffer for immunoprecipitation analysis. The lysates were centrifuged for 25 minutes at 16000 x g and the resulting supernatants were incubated for 2 hours on ice with the specific antisera, followed by immunoprecipitation using protein A-sepharose and analysis in 7% SDS-PAGE. Polypeptides were transferred to nitrocellulose and analyzed by immunoblotting using anti-phosphotyrosine (Transduction Laboratories) and anti-receptor antibodies, as described by Pajusola *et al., Oncogene, 9*:3545-3555 (1994). Filter stripping was carried out at 50°C for 30 minutes in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, with occasional agitation. The results of the experiment are shown in Fig. 33. The results demonstrate that culture medium containing mouse VEGF-C stimulates the autophosphorylation of VEGFR-3 to a similar extent as human baculoviral VEGF-C or the tyrosyl phosphatase inhibitor pervanadate.

VEGFR-2 stimulation was studied in subconfluent porcine aortic endothelial (PAE) cells expressing Kdr (VEGFR-2) (PAE-VEGFR-2) [Waltenberger *et al., J. Biol. Chem., 269*:26988-26995 (1994)], which were starved overnight in serum-free medium containing 0.2% BSA. Stimulation was carried out and the lysates prepared as described above. For receptor immunoprecipitation,

specific antiserum for VEGFR-2 [Waltenberger *et al., J. Biol. Chem., 269*:26988-26995 (1994)] was used. The immunoprecipitates were analyzed as described for VEGFR-3 in 7% SDS-PAGE followed by Western blotting with anti-phosphotyrosine antibodies, stripping of the filter, and re-probing it with anti-VEGFR-2 antibodies (Santa Cruz).

Mouse VEGF-C appeared to be a potent inducer of VEGFR-3 autophosphorylation, with the $195x10^3$ M_r precursor and proteolytically cleaved $125x10^3$ M_r tyrosine kinase polypeptides of the receptor (Pajusola *et al., Oncogene, 9*:3545-3555 (1994)), being phosphorylated. VEGFR-2 stimulation was first tried with unconcentrated medium from cells expressing recombinant VEGF-C, but immunoblotting analysis did not reveal any receptor autophosphorylation.

To further determine whether mouse recombinant VEGF-C can also induce VEGFR-2 autophosphorylation as observed for human VEGF-C, PAE cells expressing VEGFR-2 were stimulated with tenfold concentrated medium from cultures transfected with mouse VEGF-C expression vector and autophosphorylation was analyzed. For comparison, cells treated with tenfold concentrated medium containing human recombinant VEGF-C (Joukov *et al.*, (1996)), unconcentrated medium from human VEGF-C baculovirus infected insect cells, or pervanadate (a tyrosyl phosphatase inhibitor) were used. As can be seen from Fig. 33, in response to human baculoviral VEGF-C as well as pervanadate treatment, VEGFR-2 was prominently phosphorylated, whereas human and mouse recombinant VEGF-C gave a weak and barely detectable enhancement of autophosphorylation, respectively. Media from cell cultures transfected with empty vector or VEGF-C cloned in the antisense orientation did not induce autophosphorylation of VEGFR-2. Therefore, mouse VEGF-C binds to VEGFR-3 and activates this receptor at a much lower concentration than needed for the activation of VEGFR-2. Nevertheless, the invention comprehends methods

for using the materials of the invention to take advantage of the interaction of VEGF-C with VEGFR-2, in addition to the interaction between VEGF-C and VEGFR-3.

Example 27

VEGF-C E104-S213 fragment expressed in Pichia yeast stimulates autophosphorylation of Flt4 (VEGFR-3) and KDR (VEGFR-2)

A truncated form of human VEGF-C cDNA was constructed wherein (1) the sequence encoding residues of a putative mature VEGF-C amino terminus H_2N-E(104)ETIK (SEQ ID NO: 33, residues 104 et seq.) was fused in-frame to the yeast PHO1 signal sequence (Invitrogen Pichia Expression Kit, Catalog #K1710-01), and (2) a stop codon was introduced after amino acid 213 (H_2N- ...RCMS; *i.e.*, after codon 213 of SEQ ID NO: 32). The resultant truncated cDNA construct was then inserted into the *Pichia pastoris* expression vector pHIL-S1 (Invitrogen). For the cloning, an internal *Bgl*II site in the VEGF-C coding sequence was mutated without change of the encoded polypeptide sequence.

This VEGF-C expression vector was then transfected into Pichia cells and positive clones were identified by screening for the expression of VEGF-C protein in the culture medium by Western blotting. One positive clone was grown in a 50 ml culture, and induced with methanol for various periods of time from 0 to 60 hours. About 10 μl of medium was analyzed by gel electrophoresis, followed by Western blotting and detection with anti-VEGF-C antiserum, as described above. As can be seen in Figure 25, an approximately 24 kD polypeptide (note the band spreading due to glycosylation) accumulates in the culture medium of cells transfected with the recombinant VEGF-C construct, but not in the medium of mock-transfected cells or cells transfected with the vector alone.

The medium containing the recombinant VEGF-C protein was concentrated by Centricon 30 kD cutoff

ultrafiltration and used to stimulate NIH 3T3 cells expressing Flt4 (VEGFR-3) and porcine aortic endothelial (PAE) cells expressing KDR (VEGFR-2). The stimulated cells were lysed and immunoprecipitated using VEGFR-specific antisera and the immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine antibodies, chemiluminescence, and fluorography. As a positive control for maximal autophosphorylation of the VEGFRs, vanadate (VO_4) treatment of the cells for 10 minutes was used. As can be seen from the results shown in Fig. 26, medium from *Pichia* cultures secreting the recombinant VEGF-C polypeptide induces autophosphorylation of both Flt41 polypeptides of 195 kD and 125 kD as well as the KDR polypeptide of about 200 kD. Vanadate, on the other hand, induces heavy tyrosyl phosphorylation of the receptor bands in addition to other bands probably coprecipitating with the receptors.

These results demonstrate that a VEGF-homologous domain of VEGF-C consisting of amino acid residues 104E - 213S (SEQ ID NO: 33, residues 104-213) can be recombinantly produced in yeast and is capable of stimulating the autophosphorylation of Flt4 (VEGFR-3) and KDR (VEGFR-2). Recombinant VEGF-C fragments such as the fragment described herein, which are capable of stimulating Flt4 or KDR autophosphorylation are intended as aspects of the invention; methods of using these fragments are also within the scope of the invention.

Example 28

Properties of the differentially processed forms of VEGF-C

The following oligonucleotides were used to generate a set of VEGF-C variants:

5'- TCTCTTCTGTGCTTGAGTTGAG -3' (SEQ ID NO: 42), used to generate VEGF-C R102S (arginine mutated to serine at position 102 (SEQ ID NO: 33));

5'-TCTCTTCTGTCCCTGAGTTGAG -3' (SEQ ID NO: 43), used to generate VEGF-C R102G (arginine mutated to glycine at

position 102 (SEQ ID NO: 33));

5'-TGTGCTGCAGCAAATTTTATAGTCTCTTCTGTGGCGGCGGC GGCGGCGGGCGCCTCGCGAGGACC -3' (SEQ ID NO: 44), used to generate VEGF-C ΔN (deletion of N-terminal propeptide corresponding to amino acids 32-102 (SEQ ID NO: 33));

5'- CTGGCAGGGAACTGCTAATAATGGAATGAA - 3' (SEQ ID NO: 45), used to generate VEGF-C R226,227S (arginine codons mutated to serines at positions 226 and 227 (SEQ ID NO: 33));

5'-GGGCTCCGCGTCCGAGAGGTCGAGTCCGGACTCGTGATGGT GATGGTGATGGGCGGCGGCGGCGGCGGGCGCCTCGCGAGGACC -3' (SEQ ID NO: 46), used to generate VEGF-C NHis (this construct encodes a polypeptide with a 6xHis tag fused to the N-terminus of the secreted precursor (amino acid 32 of SEQ ID NO: 33)).

Some of the foregoing VEGF-C variant constructs were further modified to obtain additional constructs. For example, VEGF-C R102G in pALTER (Promega) and oligonucleotide 5'-GTATTATAATGTCCTCCACCAAATTTTATAG -3' (SEQ ID NO: 47) were used to generate VEGF-C 4G, which encodes a polypeptide with four point mutations: R102G, A110G, A111G, and A112G (alanines mutated to glycines at positions 110-112 (SEQ ID NO: 33). These four mutations are adjacent to predicted sites of cleavage of VEGF-C expressed in PC-3 and recombinantly expressed in 293 EBNA cells.

Another construct was created using VEGF-C ΔN and oligonucleotide 5'-GTTCGCTGCCTGACACTGTGGTAGTGTTGCTGGC GGCCGCTAGTGATGGTGATGGTGATGAATAATGGAATGAACTTGTCTGTAAACATCC AG -3' (SEQ ID NO: 48) to generate VEGF-C ΔNΔCHis. This construct encodes a polypeptide with a deleted N-terminal propeptide (amino acids 32-102); a deleted C-terminal propeptide (amino acids 226-419 of SEQ ID NO: 33); and an added 6xHis tag at the C-terminus.

All constructs were further digested with *Hind*III and *Not*I, subcloned into *Hind*III/*Not*I digested pREP7 vector, and used to transfect 293 EBNA cells.

About 48 hours after transfection, the cells were either metabolically labelled with Pro-mix™ as described above, or starved in serum-free medium for 2 days. Media were then collected and used in subsequent experiments. As can be seen from Figs. 27A-B, wild type (wt) VEGF-C, VEGF-C NHis and VEGF-C ΔNΔCHis were expressed to similar levels in 293 EBNA cells. At the same time, expression of the VEGF-C 4G polypeptide was considerably lower, possibly due to the changed conformation and decreased stability of the translated product. However, all the above VEGF-C variants were secreted from the cells (compare Figs. 27A and 27B). The conditioned media from the transfected and starved cells were concentrated 5-fold and used to assess their ability to stimulate tyrosine phosphorylation of Flt4 (VEGFR-3) expressed in NIH 3T3 cells and KDR (VEGFR-2) expressed in PAE cells.

Figs. 28A-B show that wild type (wt) VEGF-C, as well as all three mutant polypeptides, stimulate tyrosine phosphorylation of VEGFR-3. The most prominent stimulation is by the short mature VEGF-C ΔNΔCHis. This mutant, as well as VEGF-C NHis, also stimulated tyrosine phosphorylation of VEGFR-2. Thus, despite the fact that a major component of secreted recombinant VEGF-C is a dimer of 32/29 kD, the active part of VEGF-C responsible for its binding to VEGFR-3 and VEGFR-2 is localized between amino acids 102 and 226 (SEQ ID NO: 33) of the VEGF-C precursor. Analysis and comparison of binding properties and biological activities of these VEGF-C proteins and variants, using assays such as those described herein, will provide data concerning the significance of the observed major 32/29 kD and 21-23 kD VEGF-C processed forms. The data indicate that constructs encoding amino acid residues 103-225 of the VEGF-C precursor (SEQ ID NO: 33) generate a recombinant ligand that is functional for both VEGFR-3 and VEGFR-2.

The data from this and preceding examples demonstrate that numerous fragments of the VEGF-C

polypeptide retain biological activity. A naturally occurring VEGF-C polypeptide spanning amino acids 103-226 (or 103-227) of SEQ ID NO: 33, produced by a natural processing cleavage defining the C-terminus, has been shown to be active. Example 27 demonstrates that a fragment with residues 104-213 of SEQ ID NO: 33 retains biological activity.

In addition, data from Example 21 demonstrates that a VEGF-C polypeptide having its amino terminus at position 112 of SEQ ID NO: 33 retains activity. Additional experiments have shown that a fragment lacking residues 1-112 of SEQ ID NO: 33 retains biological activity.

In a related experiment, a stop codon was substituted for the lysine at position 214 of SEQ ID NO: 33 (SEQ ID NO: 32, nucleotides 991-993). The resulting recombinant polypeptide still was capable of inducing Flt4 autophosphorylation, indicating that a polypeptide spanning amino acid residues 113-213 of SEQ ID NO: 33 is biologically active.

Sequence comparisons of members of the VEGF family of polypeptides provides an indication that still smaller fragments of the polypeptide depicted in SEQ ID NO: 33 will retain biological activity. In particular, eight highly conserved cysteine residues of the VEGF family of polypeptides define a region from residues 131 - 211 of SEQ ID NO: 33 (see Figure 31) of evolutionary signficance; therefore, a polypeptide spanning from about residue 131 to about residue 211 is expected to retain VEGF-C biological activity. In fact, a polypeptide which retains the conserved motif RCXXCC (e.g., a polypeptide comprising from about residue 161 to about residue 211 of SEQ ID NO: 33 is postulated to retain VEGF-C biological activity. To maintain native conformation of these fragments, it may be preferred to retain about 1-2 additional amino acids at the carboxy-terminus and 1-2 or more amino acids at the amino terminus.

Beyond the preceding considerations, evidence exists that smaller fragments and/or fragment variants which lack the conserved cysteines nonetheless will retain VEGF-C biological activity. Consequently, the materials and methods of the invention include all VEGF-C fragments and variants that retain at least one biological activity of VEGF-C, regardless of the presence or absence of members of the conserved set of cysteine residues.

Example 29

Expression of human VEGF-C under the human K14 keratin promoter in transgenic mice induces abundant growth of lymphatic vessels in the skin

The Flt4 receptor tyrosine kinase is relatively specifically expressed in the endothelia of lymphatic vessels. Kaipainen *et al., Proc. Natl. Acad. Sci. (USA), 92:* 3566-3570 (1995). Furthermore, the VEGF-C growth factor stimulates the Flt4 receptor, showing less activity towards the KDR receptor of blood vessels (Joukov *et al., EMBO J., 15:* 290-298 (1996); See Example 26).

Experiments were conducted in transgenic mice to analyze the specific effects of VEGF-C overexpression in tissues. The human K14 keratin promoter is active in the basal cells of stratified squamous epithelia (Vassar *et al., Proc. Natl. Acad. Sci. (USA), 86:*1563-1567 (1989)) and was used as the expression control element in the recombinant VEGF-C transgene. The vector containing the K14 keratin promoter is described in Vassar *et al., Genes Dev., 5:*714-727 (1991) and Nelson *et al., J. Cell Biol. 97:*244-251 (1983).

The recombinant VEGF-C transgene was constructed using the human full length VEGF-C cDNA (GenBank Acc. No. X94216). This sequence was excised from a pCI-neo vector (Promega) with *Xho*I/*Not*I, and the resulting 2027 base pair fragment containing the open reading frame and stop codon (nucleotides 352-1611 of SEQ

ID NO: 32) was isolated. The isolated fragment was then subjected to an end-filling reaction using the Klenow fragment of DNA polymerase. The blunted fragment was then ligated to a similarly opened *Bam*HI restriction site in the K14 vector. The resulting construct contained the *Eco*RI site derived from the polylinker of the pCI-neo vector. This *Eco*RI site was removed using standard techniques (a Klenow-mediated fill-in reaction following partial digestion of the recombinant intermediate with *Eco*RI) to facilitate the subsequent excision of the DNA fragment to be injected into fertilized mouse oocytes. The resulting clone, designated K14-VEGF-C, is illustrated in Fig. 20.

The *Eco*RI-*Hin*dIII fragment from clone K14 VEGF-C containing the K14 promoter, VEGF-C cDNA, and K14 polyadenylation signal was isolated and injected into fertilized oocytes of the FVB-NIH mouse strain. The injected zygotes were transplanted to oviducts of pseudopregnant C57BL/6 x DBA/2J hybrid mice. The resulting founder mice were analyzed for the presence of the transgene by polymerase chain reaction of tail DNA using the primers: 5'-CATGTACGAACCGCCAG-3' (SEQ ID NO: 49) and 5'-AATGACCAGAGAGAGGCGAG-3' (SEQ ID NO: 50). In addition, the tail DNAs were subjected to *Eco*RV digestion and subsequent Southern analysis using the *Eco*RI-*Hin*dIII fragment injected into the mice. Out of 8 pups analyzed at 3 weeks of age, 2 were positive, having approximately 40-50 copies and 4-6 copies of the transgene in their respective genomes.

The mouse with the high copy number transgene was small, developed more slowly than its litter mates and had difficulty eating (*i.e.*, suckling). Further examination showed a swollen, red snout and poor fur. Although fed with a special liquid diet, it suffered from edema of the upper respiratory and digestive tracts after feeding and had breathing difficulties. This mouse died eight weeks after birth and was immediately processed for

histology, immunohistochemistry, and *in situ* hybridization.

Histological examination showed that in comparison to the skin of littermates, the dorsal dermis of K14-VEGF-C transgenic mice was atrophic and connective tissue was replaced by large lacunae devoid of red cells, but lined with a thin endothelial layer (white arrows in Figs. 29A-D). These distended vessel-like structures resembled those seen in human lymphangiomas. The number of skin adnexal organs and hair follicles were reduced. In the snout region, an increased number of vessels was also seen. Therefore, VEGF-C overexpression in the basal epidermis is capable of promoting the growth of extensive vessel structure in the underlying skin, including large vessel lacunae. The endothelial cells surrounding these lacunae contained abundant Flt4 mRNA in *in situ* hybridization (see Examples 23 and 30 for methodology). The vessel morphology indicates that VEGF-C stimulates the growth of vessels having features of lymphatic vessels. The other K14-VEGF-C transgenic mouse had a similar skin histopathology.

The foregoing *in vivo* data indicates utilities for both (i) VEGF-C polypeptides and polypeptide variants having VEGF-C biological activity, and (ii) anti-VEGF-C antibodies and VEGF-C antagonists that inhibit VEGF-C activity (*e.g.*, by binding VEGF-C or interfering with VEGF-C/receptor interactions. For example, the data indicates a therapeutic utility for VEGF-C polypeptides in patients wherein growth of lymphatic tissue may be desirable (e.g., in patients following breast cancer or other surgery where lymphatic tissue has been removed and where lymphatic drainage has therefore been compromised, resulting in swelling; or in patients suffering from elephantiasis). The data indicates a therapeutic utility for anti-VEGF-C antibody substances and VEGF-C antagonists for conditions wherein growth-inhibition of lymphatic tissue may be desirable (e.g., treatment of

lymphangiomas). Accordingly, methods of administering VEGF-C and VEGF-C variants and antagonists are contemplated as methods and materials of the invention.

Example 30

Expression of VEGF-C and Flt4 in the Developing Mouse

Embryos from a 16-day post-coitus pregnant mouse were prepared and fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned at 6 μm. The sections were placed on silanated microscope slides and treated with xylene, rehydrated, fixed for 20 minutes in 4% PFA, treated with proteinase K (7mg/ml; Merck, Darmstadt, Germany) for 5 minutes at room temperature, again fixed in 4% PFA and treated with acetic anhydride, dehydrated in solutions with increasing ethanol concentrations, dried and used for *in situ* hybridization.

In situ hybridization of sections was performed as described (Västrik *et al., J. Cell Biol., 128*:1197-1208 (1995)). A mouse VEGF-C antisense RNA probe was generated from linearized pBluescript II SK+ plasmid (Stratagene Inc.), containing a fragment corresponding to nucleotides 499-979 of mouse VEGF-C cDNA, where the noncoding region and the BR3P repeat were removed by Exonuclease III treatment. The fragment had been cloned into the *Eco*RI and *Hin*dIII sites of pBluescript II SK+. Radiolabeled RNA was synthesized using T7 RNA Polymerase and [^{35}S]-UTP (Amersham, Little Chalfont, UK). About two million cpm of the VEGF-C probe was applied per slide. After an overnight hybridization, the slides were washed first in 2x SSC and 20-30 mM DDT for 1 hour at 50°C. Treatment continued with a high stringency wash, 4x SSC and 20 mM DTT and 50% deionized formamide for 30 minutes at 65°C followed by RNase A treatment (20 μg/ml) for 30 minutes at 37°C. The high stringency wash was repeated for 45 minutes. Finally, the slides were dehydrated and dried for 30 minutes at

room temperature. The slides were dipped into photography emulsion and exposed for 4 weeks. Slides were developed using Kodak D-16 developer, counterstained with hematoxylin and mounted with Permount (FisherChemical).

For *in situ* hybridizations of Flt4 sequences, a mouse Flt4 cDNA fragment covering bp 1-192 of the published sequence (Finnerty *et al., Oncogene, 8*:2293-2298 (1993)) was used, and the above-described protocol was followed, with the following exceptions. Approximately one million cpm of the Flt4 probe were applied to each slide. The stringent washes following hybridization were performed in 1x SSC and 30 mM DTT for 105 minutes.

The figure shows photomicrographs of the hybridized sections in dark field microscopy (Figs. 36A-C) and light field microscopy (Fig. 36D). Magnifications used for photography were 4x for Figs. 36A-B and 10x for Figs. 36C-D. The transverse sections shown are from the cephalic region and the area shown for VEGF-C and FLT4 are about 14 sections apart, Flt4 being more cranially located in the embryo. In Fig. 36A (Flt4 probe), the developing nasopharyngeal cavity is in the midline in the upper, posterior part; in the anterior part of Fig. 36A is the snout with emerging fibrissal follicles and, in the midline, the forming nasal cavity. On both sides, the retinal pigment gives a false positive signal in dark field microscopy. The most prominently Flt4-hybridizing structures appear to correspond to the developing lymphatic and venous endothelium. Note that a plexus-like endothelial vascular structure surrounds the developing nasopharyngeal mucous membrane. In Fig. 36B, the most prominent signal is obtained from the posterior part of the developing nasal conchae, which in higher magnification (Figs. 36C-D) show the epithelium surrounding loose connective tissue/forming cartilage. This structure gives a strong *in situ* hybridization

signal for VEGF-C. Also in Fig. 36B, more weakly hybridizing areas can be seen around the snout, although this signal is much more homogeneous in appearance. Thus, the expression of VEGF-C is strikingly high in the developing nasal conchae.

The conchae are surrounded with a rich vascular plexus, important in nasal physiology as a source for the mucus produced by the epithelial cells and for warming inhaled air. It is suggested that VEGF-C is important in the formation of the concheal venous plexus at the mucous membranes, and that it may also regulate the permeability of the vessels needed for the secretion of nasal mucus. Possibly, VEGF-C and its derivatives, and antagonists, could be used in the regulation of the turgor of the conchal tissue and mucous membranes and therefore the diameter of the upper respiratory tract, as well as the quantity and quality of mucus produced. These factors are of great clinical significance in inflammatory (including allergic) and infectious diseases of the upper respiratory tract. Accordingly, the invention contemplates the use of the materials of the invention, including VEGF-C, Flt4, and their derivatives, in methods of diagnosing and treating inflammatory and infectious conditions affecting the upper respiratory tract, including nasal structures.

Example 31

Characterization of the exon-intron organization of the human VEGF-C gene

Two genomic DNA clones covering exons 1, 2, and 3 of the human VEGF-C gene were isolated from a human genomic DNA library using VEGF-C cDNA fragments as probes. In particular, a human genomic library in bacteriophage EMBL-3 lambda (Clontech) was screened using a PCR-generated fragment corresponding to nucleotides 629-746 of the human VEGF-C cDNA (SEQ ID NO: 32). One positive clone, designated "lambda 3," was identified, and the insert was subcloned as a 14 kb *Xho*I fragment

into the pBSK II vector (Stratagene). The genomic library also was screened with a labeled 130 bp *Not*I-*Sac*I fragment from the 5'-noncoding region of the VEGF-C cDNA (the *Not*I site is in the polylinker of the cloning vector; the *Sac*I site corresponds to nucleotides 92-97 of SEQ ID NO: 32). Two positive clones, designated "lambda 5" and "lambda 8," were obtained. Restriction mapping analysis showed that clone lambda 3 contains exons 2 and 3, while clone lambda 5 contains exon 1 and the putative promoter region.

Three genomic fragments containing exons 4, 5, 6 and 7 were subcloned from a genomic VEGF-C P1 plasmid clone. In particular, purified DNA from a genomic P1 plasmid clone 7660 (Paavonen *et al.*, *Circulation, 93*: 1079-1082 (1996)) was used. *Eco*RI fragments of the P1 insert DNA were ligated into pBSK II vector. Subclones of clone 7660 which contained human VEGF-C cDNA homologous sequences were identified by colony hybridization, using the full-length VEGF-C cDNA as a probe. Three different genomic fragments were identified and isolated, which contained the remaining exons 4-7.

To determine the genomic organization, the clones were mapped using restriction endonuclease cleavage. Also, the coding regions and exon-intron junctions were partially sequenced. The result of this analysis is depicted in Figures 11 and 17. The sequences of all intron-exon boundaries (Fig. 17, SEQ ID NOs: 57-68) conformed to the consensus splicing signals (Mount, *Nucl. Acids Res., 10*: 459-472 (1982)). The length of the intron between exon 5 and 6 was determined directly by nucleotide sequencing and found to be 301 bp. The length of the intron between exons 2 and 3 was determined by restriction mapping and Southern hybridization and was found to be about 1.6 kb. Each of the other introns was over 10 kb in length.

A similar analysis was performed for the murine genomic VEGF-C gene. The sequences of murine VEGF-C

intron-exon boundaries are depicted in Figure 17 and SEQ ID NOs: 69-80.

The restriction mapping and sequencing data indicated that the signal sequence and the first residues of the N-terminal propeptide are encoded by exon 1. The second exon encodes the carboxy-terminal portion of the N-terminal propeptide and the amino terminus of the VEGF homology domain. The most conserved sequences of the VEGF homology domain are distributed in exons 3 (containing 6 conserved cysteine residues) and 4 (containing 2 cys residues). The remaining exons encode cysteine-rich motifs of the type C-6X-C-10X-CRC (exons 5 and 7) and a fivefold repeated motif of type C-6X-B-3X-C-C-C, which is typical of a silk protein.

To further characterize the VEGF-C gene promoter, the lambda 5 clone was further analyzed. Restriction mapping of this clone using a combination of single- and double-digestions and Southern hybridizations indicated that it includes: (1) an approximately 5 kb region upstream of the putative initiator ATG codon, (2) exon 1, and (3) part of intron I of the VEGF-C gene.

A 3.7 kb *Xba* I fragment of clone lambda 5, containing exon 1 and 5′ and 3′ flanking sequences, was subcloned and further analyzed. As reported previously, a major VEGF-C mRNA band migrates at a position of about 2.4 kb. Calculating from the VEGF-C coding sequence of 1257 bp and a 391 bp 3′ noncoding sequence plus a polyA sequence of about 50-200 bp, the mRNA start site should be located about 550-700 bp upstream of the translation initiation codon.

To further characterize the promoter of the human VEGF-C gene, a genomic clone encompassing about 1.4 kb upstream of the translation initiation site was isolated, and the 5′ noncoding cDNA sequence and putative promoter region were sequenced. The sequence obtained is set forth in SEQ ID NO: 54. Similar to what has been observed with the VEGF gene, the VEGF-C promoter is rich

in G and C residues and lacks consensus TATA and CCAAT sequences. Instead, it has numerous putative binding sites for Sp1, a ubiquitous nuclear protein that can initiate transcription of TATA-less genes. See Pugh and Tjian, *Genes and Dev.*, *5*:105-119 (1991). In addition, sequences upstream of the VEGF-C translation start site were found to contain frequent consensus binding sites for the AP-2 factor. This suggests that the cAMP-dependent protein kinase and protein kinase C, as activators of AP-2 transcription factor [Curran and Franza, *Cell*, *55*:395-397 (1988)], mediate VEGF-C transcriptional regulation.

The VEGF-C gene is abundantly expressed in adult human tissues, such as heart, placenta, ovary and small intestine, and is induced by a variety of factors. Indeed, several potential binding sites for regulators of tissue-specific gene expression, like NFkB and GATA, are located in the distal part of the VEGF-C promoter. For example, NFkB is known to regulate the expression of tissue factor in endothelial cells. Also, transcription factors of the GATA family are thought to regulate cell-type specific gene expression.

Unlike VEGF, the VEGF-C gene does not contain a binding site for the hypoxia-inducible factor, HIF-1 (Levy *et al.*, *J. Biol. Chem.*, *270:* 13333-13340 (1995)). This finding suggests that if the VEGF-C mRNA is regulated by hypoxia, the mechanism would be based mainly on the regulation of mRNA stability. In this regard, numerous studies have shown that the major control point for the hypoxic induction of the VEGF gene is the regulation of the steady-state level of mRNA. See Levy *et al.*, *J. Biol. Chem.*, *271*: 2746-2753 (1996). The relative rate of VEGF mRNA stability and decay is considered to be determined by the presence of specific sequence motifs in its 3′ untranslated region (UTR), which have been demonstrated to regulate mRNA stability (Chen and Shyu, *Mol. Cell Biol.*, *14*: 8471-8482 (1994)). The 3′-UTR of the VEGF-C gene also contains a putative

motif of this type (TTATTT), at positions 1873-1878 of SEQ ID NO: 32.

Example 32

Identification of a VEGF-C splice variant

As reported in Example 16, a major 2.4 kb VEGF-C mRNA and smaller amounts of a 2.0 kb mRNA are observable. To clarify the origin of these RNAs, several additional VEGF-C cDNAs were isolated and characterized. A human fibrosarcoma cDNA library from HT1080 cells in the lambda gt11 vector (Clontech, product #HL1048b) was screened using a 153 bp human VEGF-C cDNA fragment as a probe as described in Example 10. See also Joukov *et al., EMBO J., 15*:290-298 (1996). Nine positive clones were picked and analyzed by PCR amplification using oligonucleotides 5'-CACGGCTTATGCAAGCAAAG-3' (SEQ ID NO: 55) and 5'-AACACAGTTTTCCATAATAG-3' (SEQ ID NO: 56) These oligonucleotides were selected to amplify the portion of the VEGF-C cDNA corresponding to nucleotides 495-1661 of SEQ ID NO: 32. PCR was performed using an annealing temperature of 55°C and 25 cycles.

The resultant PCR products were electrophoresed on agarose gels. Five clones out of the nine analyzed generated PCR fragments of the expected length of 1147 base pairs, whereas one was slightly shorter. The shorter fragment and one of the fragments of expected length were cloned into the pCRTMII vector (Invitrogen) and analyzed by sequencing. The sequence revealed that the shorter PCR fragment had a deletion of 153 base pairs, corresponding to nucleotides 904 to 1055 of SEQ ID NO: 32. These deleted bases correspond to exon 4 of the human and mouse VEGF-C genes, schematically depicted in Fig. 17. Deletion of exon 4 results in a frameshift, which in turn results in a C-terminal truncation of the full-length VEGF-C precursor, with fifteen amino acid residues translated from exon 5 in a different frame than the frame used to express the full-length protein. Thus,

the C-terminal amino acid sequence of the resulting truncated polypeptide would be --Leu (181)-Ser-Lys-Thr-Val-Ser-Gly-Ser-Glu-Gln-Asp-Leu-Pro-His-Glu-Leu-His-Val-Glu(199) (SEQ ID NO: 81). The VEGF-C variant encoded by this splice variant would not contain the C-terminal cleavage site of the VEGF-C precursor. Thus, a putative alternatively spliced RNA form lacking conserved exon 4 was identified in HT-1080 fibrosarcoma cells and this form is predicted to encode a protein of 199 amino acid residues, which could be an antagonist of VEGF-C.

Example 33

VEGF-C is similarly processed in different cell cultures in vitro

To study whether VEGF-C is similarly processed in different cell types, 293 EBNA cells, COS-1 cells and HT-1080 cells were transfected with wild type human VEGF-C cDNA and labelled with Pro-Mix™ as described in Example 22. The conditioned media from the cultures were collected and subjected to immunoprecipitation using antiserum 882 (described in Example 21, recognizing a peptide corresponding to amino acids 104-120 of SEQ ID NO: 33). The immunoprecipitated polypeptides were separated via SDS-PAGE, and detected via autoradiography. The major form of secreted recombinant VEGF-C observed from all cell lines tested is a 29/32 kD doublet. These two polypeptides are bound to each other by disulfide bonds, as described in Example 22. A less prominent band of approximately 21 kD also was detected in the culture media. Additionally, a non-processed VEGF-C precursor of 63 kDa was observed. This form was more prominent in the COS-1 cells, suggesting that proteolytic processing of VEGF-C in COS cells is less efficient than in 293 EBNA cells. Endogenous VEGF-C (in non-transfected cells) was not detectable under these experimental conditions in the HT-1080 cells, but was readily detected in the conditioned medium of the PC-3 cells. Analysis of the subunit

polypeptide sizes and ratios in PC-3 cells and 293 EBNA cells revealed strikingly similar results: the most prominent form was a doublet of 29/32 kDa and a less prominent form the 21 kD polypeptide. The 21 kD form produced by 293 EBNA cells was not recognized by the 882 antibody in the Western blot, although it is recognized when the same antibody is used for immunoprecipitation (see data in previous examples). As reported in Example 21, cleavage of the 32 kD form in 293 EBNA cells occurs between amino acid residues 111 and 112 (SEQ ID NO: 33), downstream of the cleavage site in PC-3 cells (between residues 102 and 103). Therefore, the 21 kD form produced in 293 EBNA cells does not contain the complete N-terminal peptide used to generate antiserum 882.

In a related experiment, PC-3 cells were cultured in serum-free medium for varying periods of time (1 - 8 days) prior to isolation of the conditioned medium. The conditioned medium was concentrated using a Centricon device (Amicon, Beverly, USA) and subjected to Western blotting analysis using antiserum 882. After one day of culturing, a prominent 32 kD band was detected. Increasing amounts of a 21-23 kD form were detected in the conditioned media from 4 day and 8 day cultures. The diffuse nature of this polypeptide band, which is simply called the 23 kD polypeptide in example 5 and several subsequent examples is most likely due to a heterogenous and variable amount of glycosylation. These results indicate that, initially, the cells secrete a 32 kD polypeptide, which is further processed or cleaved in the medium to yield the 21-23 kD form. The microheterogeneity of this polypeptide band would then arise from the variable glycosylation degree and, from microheterogeneity of the processing cleavage sites, such as obtained for the amino terminus in PC-3 and 293 EBNA cell cultures. The carboxyl terminal cleavage site could also vary, examples of possible cleavage sites would be between residues 225-226, 226-227 and 227-228 as well as between residues 216-217.

Taken together, these data suggest the possibility that secreted cellular protease(s) are responsible for the generation of the 21-23 kD form of VEGF-C from the 32 kD polypeptide. Such proteases could be used in vitro to cleave VEGF-C precursor proteins in solution during the production of VEGF-C, or used in cell culture and in vivo to release biologically active VEGF-C.

Example 34

Differential binding of VEGF-C forms by the extracellular domains of VEGFR-3 and VEGFR-2

In two parallel experiments, 293 EBNA cells were transfected with a construct encoding recombinant wild type VEGF-C or a construct encoding VEGF-C DNDCHis (Example 28) and about 48 hours after transfection, metabolically labelled with Pro-Mix™ as described in previous examples. The media were collected from mock-transfected and transfected cells and used for receptor binding analyses.

Receptor binding was carried out in binding buffer (PBS, 0.5% BSA, 0.02% Tween 20, 1 microgram/ml heparin) containing approximately 0.2 microgram of either (a) a fusion protein comprising a VEGFR-3 extracellular domain fused to an immunoglobulin sequence (VEGFR-3-Ig) or (b) a fusion protein comprising VEGFR-2 extracellular domain fused to an alkaline phosphatase sequence (VEGF-R-2-AP; Cao *et al.*, *J. Biol. Chem.* *271*:3154-62 (1996)). As a control, similar aliquots of the 293 EBNA conditioned media were mixed with 2 µl of anti-VEGF-C antiserum (VEGF-C IP).

After incubation for 2 hours at room temperature, anti-VEGF-C antibodies and VEGFR-3-Ig protein were adsorbed to protein A-sepharose (PAS) and VEGFR-2-AP was immunoprecipitated using anti-AP monoclonal antibodies (Medix Biotech, Genzyme Diagnostics, San Carlos, CA, USA) and protein G-sepharose. Complexes containing VEGF-C bound to VEGFR-3-Ig or VEGFR-2-AP were washed three times

in binding buffer, twice in 20 mM Tris-HCl (pH 7.4) and VEGF-C immunoprecipitates were washed three times in RIPA buffer and twice in 20 mM tris-HCl (pH 7.4) and analyzed via SDS-PAGE under reducing and nonreducing conditions. As a control, the same media were precipitated with anti-AP and protein G-sepharose (PGS) or with PAS to control for possible nonspecific adsorption.

These experiments revealed that VEGFR-3 bound to both the 32/29 kD and 21-23 kD forms of recombinant VEGF-C, whereas VEGFR-2 bound preferentially to the 21-23 kD component from the conditioned media. In addition, small amounts of 63 kD and 52 kD VEGF-C forms were observed binding with VEGFR-3. Further analysis under nonreducing conditions indicates that a great proportion of the 21-23 kD VEGF-C bound to either receptor does not contain interchain disulfide bonds. These findings reinforce the results that VEGF-C binds VEGFR-2. This data suggests a utility for recombinant forms of VEGF-C which are active towards VEGFR-3 only or which are active towards both VEGFR-3 and VEGFR-2. On the other hand, these results, together with the results in Example 28, do not eliminate the possibility that the 32/29 kD dimer binds VEGFR-3 but does not activate it. The failure of the 32/29 kD dimer to activate VEGFR-3 could explain the finding that conditioned medium from the N-His VEGF-C transfected cells induced a less prominent tyrosine phosphorylation of VEGFR-3 than medium from VEGF-C DNDCHis transfected cells, even though expression of the former polypeptide was much higher (see Figs. 27 and 28). Stable VEGF-C polypeptide variants that bind to a VEGF-C receptor but fail to activate the receptor are useful as VEGF-C antagonists.

Deposit of Biological Materials: Plasmid FLT4-L has been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Dr., Rockville MD 20952 (USA), pursuant to the provisions of the Budapest Treaty, and has been assigned a deposit date of 24 July 1995 and ATCC accession number 97231.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those in the art. Accordingly, only such limitations as appear in the appended claims should be placed on the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Helsinki University Licensing Ltd Oy

(ii) TITLE OF INVENTION: Receptor Ligand VEGF-C

(iii) NUMBER OF SEQUENCES: 81

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Oy Jalo Ant-Wuorinen Ab
(B) STREET: Iso Roobertinkatu 4-6 A
(C) CITY: Helsinki
(E) COUNTRY: Finland
(F) ZIP: 00120

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/671,573
(B) FILING DATE: 28-JUN-1996

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/601,132
(B) FILING DATE: 14-FEB-1996

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/585,895
(B) FILING DATE: 12-JAN-1996

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/510,133
(B) FILING DATE: 01-AUG-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Karvinen, Leena
(C) REFERENCE/DOCKET NUMBER: 28999

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +358 0 648 606
(B) TELEFAX: +358 0 640 575
(C) TELEX: 123505 JALO FI

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGTCCTCGCT GTCCTTGTCT 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACATGCATGC CACCATGCAG CGGGGCGCCG CGCTGTGCCT GCGACTGTGG CTCTGCCTGG 60

GACTCCTGGA 70

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACATGCATGC CCCGCCGGTC ATCC 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGAATTCCC CATGACCCCA AC 22

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCATCGATGG ATCCTACCTG AAGCCGCTTT CTT 33

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATTTAGGTGA CACTATA 17

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCATCGATGG ATCCCGATGC TGCTTAGTAG CTGT 34

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Pro Met Thr Pro Thr Thr Tyr Lys Gly Ser Val Asp Asn Gln Thr Asp
1 5 10 15

Ser Gly Met Val Leu Ala Ser Glu Glu Phe Glu Gln Ile Glu Ser Arg
20 25 30

His Arg Gln Glu Ser Gly Phe Arg
35 40

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGGAGTCGA CTTGGCGGAC T 21

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCGGATCCC TAGTGATGGT GATGGTGATG TCTACCTTCG ATCATGCTGC CCTTATCCTC 60

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCAAGCTTG GATCCAAGTG GCTACTCCAT GACC 34

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTTGCCTGTG ATGTGCACCA 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Xaa Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr Asn Thr Glu Ile
1 5 10 15

Leu Lys

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCAGARGARA CNATHAA 17

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Glu Glu Thr Ile Lys
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCAYTTNARD ATYTCNGT 18

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Thr Glu Ile Leu Lys
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATTCGCTGCA GCACACTACA AC 22

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCNGTGTTGT AGTGTGCTG 19

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ala Ala His Tyr Asn Thr Glu

1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TAATACGACT CACTATAGGG 20

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GTTGTAGTGT GCTGCAGCGA ATTT 24

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Lys Phe Ala Ala Ala His Tyr Asn
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TCACTATAGG GAGACCCAAG C 21

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 219 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCACTATAGG GAGACCCAAG CTTGGTACCG AGCTCGGATC CACTAGTAAC GGCCGCCAGT 60

GTGGTGGAAT TCGACGAACT CATGACTGTA CTCTACCCAG AATATTGGAA AATGTACAAG 120

TGTCAGCTAA GGCAAGGAGG CTGGCAACAT AACAGAGAAC AGGCCAACCT CAACTCAAGG 180

ACAGAAGAGA CTATAAAATT CGCTGCAGCA CACTACAAC 219

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ACAGAGAACA GGCCAACC 18

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCTAGCATTT AGGTGACAC 19

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AAGAGACTAT AAAATTCGCT GCAGC 25

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCCTCTAGAT GCATGCTCGA 20

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTTGTAGTGT GCTGCAGCGA ATTT 24

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TCACTATAGG GAGACCCAAG C 21

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1997 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 352..1608

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
CCCGCCCCGC CTCTCCAAAA AGCTACACCG ACGCGGACCG CGGCGGCGTC CTCCCTCGCC      60

CTCGCTTCAC CTCGCGGGCT CCGAATGCGG GGAGCTCGGA TGTCCGGTTT CCTGTGAGGC     120

TTTTACCTGA CACCCGCCGC CTTTCCCCGG CACTGGCTGG GAGGGCGCCC TGCAAAGTTG     180

GGAACGCGGA GCCCCGGACC CGCTCCCGCC GCCTCCGGCT CGCCCAGGGG GGGTCGCCGG     240

GAGGAGCCCG GGGGAGAGGG ACCAGGAGGG GCCCGCGGCC TCGCAGGGGC GCCCGCGCCC     300

CCACCCCTGC CCCCGCCAGC GGACCGGTCC CCCACCCCCG GTCCTTCCAC C ATG CAC      357
                                                          Met His
                                                            1

TTG CTG GGC TTC TTC TCT GTG GCG TGT TCT CTG CTC GCC GCT GCG CTG      405
Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala Ala Leu
          5                  10                  15

CTC CCG GGT CCT CGC GAG GCG CCC GCC GCC GCC GCC GCC TTC GAG TCC      453
Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Ala Phe Glu Ser
     20                  25                  30

GGA CTC GAC CTC TCG GAC GCG GAG CCC GAC GCG GGC GAG GCC ACG GCT      501
Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala Thr Ala
 35                  40                  45                  50

TAT GCA AGC AAA GAT CTG GAG GAG CAG TTA CGG TCT GTG TCC AGT GTA      549
Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Ser Val
                 55                  60                  65
```

```
GAT GAA CTC ATG ACT GTA CTC TAC CCA GAA TAT TGG AAA ATG TAC AAG          597
Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Tyr Lys
               70                  75                  80

TGT CAG CTA AGG AAA GGA GGC TGG CAA CAT AAC AGA GAA CAG GCC AAC          645
Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln Ala Asn
            85                  90                  95

CTC AAC TCA AGG ACA GAA GAG ACT ATA AAA TTT GCT GCA GCA CAT TAT          693
Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr
        100                 105                 110

AAT ACA GAG ATC TTG AAA AGT ATT GAT AAT GAG TGG AGA AAG ACT CAA          741
Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln
    115                 120                 125                 130

TGC ATG CCA CGG GAG GTG TGT ATA GAT GTG GGG AAG GAG TTT GGA GTC          789
Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val
                135                 140                 145

GCG ACA AAC ACC TTC TTT AAA CCT CCA TGT GTG TCC GTC TAC AGA TGT          837
Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys
            150                 155                 160

GGG GGT TGC TGC AAT AGT GAG GGG CTG CAG TGC ATG AAC ACC AGC ACG          885
Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr
        165                 170                 175

AGC TAC CTC AGC AAG ACG TTA TTT GAA ATT ACA GTG CCT CTC TCT CAA          933
Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln
    180                 185                 190

GGC CCC AAA CCA GTA ACA ATC AGT TTT GCC AAT CAC ACT TCC TGC CGA          981
Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg
195                 200                 205                 210

TGC ATG TCT AAA CTG GAT GTT TAC AGA CAA GTT CAT TCC ATT ATT AGA         1029
Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg
                215                 220                 225

CGT TCC CTG CCA GCA ACA CTA CCA CAG TGT CAG GCA GCG AAC AAG ACC         1077
Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr
            230                 235                 240

TGC CCC ACC AAT TAC ATG TGG AAT AAT CAC ATC TGC AGA TGC CTG GCT         1125
Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala
        245                 250                 255

CAG GAA GAT TTT ATG TTT TCC TCG GAT GCT GGA GAT GAC TCA ACA GAT         1173
Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp
    260                 265                 270

GGA TTC CAT GAC ATC TGT GGA CCA AAC AAG GAG CTG GAT GAA GAG ACC         1221
Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr
275                 280                 285                 290

TGT CAG TGT GTC TGC AGA GCG GGG CTT CGG CCT GCC AGC TGT GGA CCC         1269
Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro
                295                 300                 305

CAC AAA GAA CTA GAC AGA AAC TCA TGC CAG TGT GTC TGT AAA AAC AAA         1317
His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys
            310                 315                 320

CTC TTC CCC AGC CAA TGT GGG GCC AAC CGA GAA TTT GAT GAA AAC ACA         1365
Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr
        325                 330                 335
```

```
TGC CAG TGT GTA TGT AAA AGA ACC TGC CCC AGA AAT CAA CCC CTA AAT      1413
Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn
    340                 345                 350

CCT GGA AAA TGT GCC TGT GAA TGT ACA GAA AGT CCA CAG AAA TGC TTG      1461
Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu
355                 360                 365                 370

TTA AAA GGA AAG AAG TTC CAC CAC CAA ACA TGC AGC TGT TAC AGA CGG      1509
Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg
                375                 380                 385

CCA TGT ACG AAC CGC CAG AAG GCT TGT GAG CCA GGA TTT TCA TAT AGT      1557
Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser
            390                 395                 400

GAA GAA GTG TGT CGT TGT GTC CCT TCA TAT TGG AAA AGA CCA CAA ATG      1605
Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met
        405                 410                 415

AGC TAAGATTGTA CTGTTTTCCA GTTCATCGAT TTTCTATTAT GGAAAACTGT           1658
Ser

GTTGCCACAG TAGAACTGTC TGTGAACAGA GAGACCCTTG TGGGTCCATG CTAACAAAGA   1718

CAAAAGTCTG TCTTTCCTGA ACCATGTGGA TAACTTTACA GAAATGGACT GGAGCTCATC   1778

TGCAAAAGGC CTCTTGTAAA GACTGGTTTT CTGCCAATGA CCAAACAGCC AAGATTTTCC   1838

TCTTGTGATT TCTTTAAAAG AATGACTATA TAATTTATTT CCACTAAAAA TATTGTTTCT   1898

GCATTCATTT TTATAGCAAC AACAATTGGT AAAACTCACT GTGATCAATA TTTTTATATC   1958

ATGCAAAATA TGTTTAAAAT AAAATGAAAA TTGTATTAT                          1997
```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 419 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Met His Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala
 1               5                  10                  15

Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Ala Phe
            20                  25                  30

Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala
        35                  40                  45

Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser
    50                  55                  60

Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met
65                  70                  75                  80

Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln
                85                  90                  95

Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala
            100                 105                 110

His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys
        115                 120                 125
```

Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe
130 135 140

Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr
145 150 155 160

Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr
165 170 175

Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu
180 185 190

Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser
195 200 205

Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile
210 215 220

Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn
225 230 235 240

Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys
245 250 255

Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser
260 265 270

Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu
275 280 285

Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys
290 295 300

Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys
305 310 315 320

Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu
325 330 335

Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro
340 345 350

Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys
355 360 365

Cys Leu Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr
370 375 380

Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser
385 390 395 400

Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro
405 410 415

Gln Met Ser

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TGAGTGATTTGTAGCTGCTGTG 22

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TATTGCAGCAACCCCCACATCT 22

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4416 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCACGCGCAG CGGCCGGAGA TGCAGCGGGG CGCCGCGCTG TGCCTGCGAC TGTGGCTCTG 60

CCTGGGACTC CTGGACGGCC TGGTGAGTGG CTACTCCATG ACCCCCCCGA CCTTGAACAT 120

CACGGAGGAG TCACACGTCA TCGACACCGG TGACAGCCTG TCCATCTCCT GCAGGGGACA 180

GCACCCCCTC GAGTGGGCTT GGCCAGGAGC TCAGGAGGCG CCAGCCACCG GAGACAAGGA 240

CAGCGAGGAC ACGGGGGTGG TGCGAGACTG CGAGGGCACA GACGCCAGGC CCTACTGCAA 300

GGTGTTGCTG CTGCACGAGG TACATGCCAA CGACACAGGC AGCTACGTCT GCTACTACAA 360

GTACATCAAG GCACGCATCG AGGGCACCAC GGCCGCCAGC TCCTACGTGT TCGTGAGAGA 420

CTTTGAGCAG CCATTCATCA ACAAGCCTGA CACGCTCTTG GTCAACAGGA AGGACGCCAT 480

GTGGGTGCCC TGTCTGGTGT CCATCCCCGG CCTCAATGTC ACGCTGCGCT CGCAAAGCTC 540

GGTGCTGTGG CCAGACGGGC AGGAGGTGGT GTGGGATGAC CGGCGGGGCA TGCTCGTGTC 600

CACGCCACTG CTGCACGATG CCCTGTACCT GCAGTGCGAG ACCACCTGGG GAGACCAGGA 660

CTTCCTTTCC AACCCCTTCC TGGTGCACAT CACAGGCAAC GAGCTCTATG ACATCCAGCT 720

GTTGCCCAGG AAGTCGCTGG AGCTGCTGGT AGGGGAGAAG CTGGTCCTGA ACTGCACCGT 780

GTGGGCTGAG TTTAACTCAG GTGTCACCTT TGACTGGGAC TACCCAGGGA AGCAGGCAGA 840

GCGGGGTAAG TGGGTGCCCG AGCGACGCTC CCAGCAGACC CACACAGAAC TCTCCAGCAT 900

CCTGACCATC CACAACGTCA GCCAGCACGA CCTGGGCTCG TATGTGTGCA AGGCCAACAA 960

CGGCATCCAG CGATTTCGGG AGAGCACCGA GGTCATTGTG CATGAAAATC CCTTCATCAG 1020

CGTCGAGTGG CTCAAAGGAC CCATCCTGGA GGCCACGGCA GGAGACGAGC TGGTGAAGCT 1080

GCCCGTGAAG CTGGCAGCGT ACCCCCCGCC CGAGTTCCAG TGGTACAAGG ATGGAAAGGC 1140

ACTGTCCGGG CGCCACAGTC CACATGCCCT GGTGCTCAAG GAGGTGACAG AGGCCAGCAC 1200

AGGCACCTAC ACCCTCGCCC TGTGGAACTC CGCTGCTGGC CTGAGGCGCA ACATCAGCCT 1260

GGAGCTGGTG GTGAATGTGC CCCCCCAGAT ACATGAGAAG GAGGCCTCCT CCCCCAGCAT 1320

CTACTCGCGT CACAGCCGCC AGGCCCTCAC CTGCACGGCC TACGGGGTGC CCCTGCCTCT 1380

CAGCATCCAG TGGCACTGGC GGCCCTGGAC ACCCTGCAAG ATGTTTGCCC AGCGTAGTCT 1440

CCGGCGGCGG CAGCAGCAAG ACCTCATGCC ACAGTGCCGT GACTGGAGGG CGGTGACCAC 1500

GCAGGATGCC GTGAACCCCA TCGAGAGCCT GGACACCTGG ACCGAGTTTG TGGAGGGAAA 1560

GAATAAGACT GTGAGCAAGC TGGTGATCCA GAATGCCAAC GTGTCTGCCA TGTACAAGTG 1620

TGTGGTCTCC AACAAGGTGG GCCAGGATGA GCGGCTCATC TACTTCTATG TGACCACCAT 1680

CCCCGACGGC TTCACCATCG AATCCAAGCC ATCCGAGGAG CTACTAGAGG GCCAGCCGGT 1740

GCTCCTGAGC TGCCAAGCCG ACAGCTACAA GTACGAGCAT CTGCGCTGGT ACCGCCTCAA 1800

CCTGTCCACG CTGCACGATG CGCACGGGAA CCCGCTTCTG CTCGACTGCA AGAACGTGCA 1860

TCTGTTCGCC ACCCCTCTGG CCGCCAGCCT GGAGGAGGTG GCACCTGGGG CGCGCCACGC 1920

CACGCTCAGC CTGAGTATCC CCCGCGTCGC GCCCGAGCAC GAGGGCCACT ATGTGTGCGA 1980

AGTGCAAGAC CGGCGCAGCC ATGACAAGCA CTGCCACAAG AAGTACCTGT CGGTGCAGGC 2040

CCTGGAAGCC CCTCGGCTCA CGCAGAACTT GACCGACCTC CTGGTGAACG TGAGCGACTC 2100

GCTGGAGATG CAGTGCTTGG TGGCCGGAGC GCACGCGCCC AGCATCGTGT GGTACAAAGA 2160

CGAGAGGCTG CTGGAGGAAA AGTCTGGAGT CGACTTGGCG GACTCCAACC AGAAGCTGAG 2220

CATCCAGCGC GTGCGCGAGG AGGATGCGGG ACGCTATCTG TGCAGCGTGT GCAACGCCAA 2280

GGGCTGCGTC AACTCCTCCG CCAGCGTGGC CGTGGAAGGC TCCGAGGATA AGGGCAGCAT 2340

GGAGATCGTG ATCCTTGTCG GTACCGGCGT CATCGCTGTC TTCTTCTGGG TCCTCCTCCT 2400

CCTCATCTTC TGTAACATGA GGAGGCCGGC CCACGCAGAC ATCAAGACGG GCTACCTGTC 2460

CATCATCATG GACCCCGGGG AGGTGCCTCT GGAGGAGCAA TGCGAATACC TGTCCTACGA 2520

TGCCAGCCAG TGGGAATTCC CCCGAGAGCG GCTGCACCTG GGGAGAGTGC TCGGCTACGG 2580

CGCCTTCGGG AAGGTGGTGG AAGCCTCCGC TTTCGGCATC CACAAGGGCA GCAGCTGTGA 2640

CACCGTGGCC GTGAAAATGC TGAAAGAGGG CGCCACGGCC AGCGAGCACC GCGCGCTGAT 2700

GTCGGAGCTC AAGATCCTCA TTCACATCGG CAACCACCTC AACGTGGTCA ACCTCCTCGG 2760

GGCGTGCACC AAGCCGCAGG GCCCCCTCAT GGTGATCGTG GAGTTCTGCA AGTACGGCAA 2820

CCTCTCCAAC TTCCTGCGCG CCAAGCGGGA CGCCTTCAGC CCCTGCGCGG AGAAGTCTCC 2880

CGAGCAGCGC GGACGCTTCC GCGCCATGGT GGAGCTCGCC AGGCTGGATC GGAGGCGGCC 2940

GGGGAGCAGC GACAGGGTCC TCTTCGCGCG GTTCTCGAAG ACCGAGGGCG GAGCGAGGCG 3000

GGCTTCTCCA GACCAAGAAG CTGAGGACCT GTGGCTGAGC CCGCTGACCA TGGAAGATCT 3060

TGTCTGCTAC AGCTTCCAGG TGGCCAGAGG GATGGAGTTC CTGGCTTCCC GAAAGTGCAT 3120

CCACAGAGAC CTGGCTGCTC GGAACATTCT GCTGTCGGAA AGCGACGTGG TGAAGATCTG 3180

TGACTTTGGC CTTGCCCGGG ACATCTACAA AGACCCTGAC TACGTCCGCA AGGGCAGTGC 3240

CCGGCTGCCC CTGAAGTGGA TGGCCCCTGA AAGCATCTTC GACAAGGTGT ACACCACGCA 3300

GAGTGACGTG TGGTCCTTTG GGGTGCTTCT CTGGGAGATC TTCTCTCTGG GGGCCTCCCC 3360

GTACCCTGGG GTGCAGATCA ATGAGGAGTT CTGCCAGCGG CTGAGAGACG GCACAAGGAT 3420

GAGGGCCCCG GAGCTGGCCA CTCCCGCCAT ACGCCGCATC ATGCTGAACT GCTGGTCCGG 3480

AGACCCCAAG GCGAGACCTG CATTCTCGGA GCTGGTGGAG ATCCTGGGGG ACCTGCTCCA 3540

GGGCAGGGGC CTGCAAGAGG AAGAGGAGGT CTGCATGGCC CCGCGCAGCT CTCAGAGCTC 3600

AGAAGAGGGC AGCTTCTCGC AGGTGTCCAC CATGGCCCTA CACATCGCCC AGGCTGACGC 3660

TGAGGACAGC CCGCCAAGCC TGCAGCGCCA CAGCCTGGCC GCCAGGTATT ACAACTGGGT 3720

GTCCTTTCCC GGGTGCCTGG CCAGAGGGGC TGAGACCCGT GGTTCCTCCA GGATGAAGAC 3780

ATTTGAGGAA TTCCCCATGA CCCCAACGAC CTACAAAGGC TCTGTGGACA ACCAGACAGA 3840

CAGTGGGATG GTGCTGGCCT CGGAGGAGTT TGAGCAGATA GAGAGCAGGC ATAGACAAGA 3900

AAGCGGCTTC AGGTAGCTGA AGCAGAGAGA GAGAAGGCAG CATACGTCAG CATTTTCTTC 3960

TCTGCACTTA TAAGAAAGAT CAAAGACTTT AAGACTTTCG CTATTTCTTC TACTGCTATC 4020

TACTACAAAC TTCAAAGAGG AACCAGGAGG ACAAGAGGAG CATGAAAGTG GACAAGGAGT 4080

GTGACCACTG AAGCACCACA GGGAAGGGGT TAGGCCTCCG GATGACTGCG GGCAGGCCTG 4140

GATAATATCC AGCCTCCCAC AAGAAGCTGG TGGAGCAGAG TGTTCCCTGA CTCCTCCAAG 4200

GAAAGGGAGA CGCCCTTTCA TGGTCTGCTG AGTAACAGGT GCNTTCCCAG ACACTGGCGT 4260

TACTGCTTGA CCAAAGAGCC CTCAAGCGGC CCTTATGCCA GCGTGACAGA GGGCTCACCT 4320

CTTGCCTTCT AGGTCACTTC TCACACAATG TCCCTTCAGC ACCTGACCCT GTGCCCGCCA 4380

GTTATTCCTT GGTAATATGA GTAATACATC AAAGAG 4416

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4273 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

AAGCTTATCG ATTTCGAACC CGGGGGTACC GAATTCCTCG AGTCTAGAGG AGCATGCCTG 60

CAGGTCGACC GGGCTCGATC CCCTCGCGAG TTGGTTCAGC TGCTGCCTGA GGCTGGACGA 120

CCTCGCGGAG TTCTACCGGC AGTGCAAATC CGTCGGCATC CAGGAAACCA GCAGCGGCTA 180

TCCGCGCATC CATGCCCCCG AACTGCAGGA GTGGGGAGGC ACGATGGCCG CTTTGGTCCC 240

GGATCTTTGT GAAGGAACCT TACTTCTGTG GTGTGACATA ATTGGACAAA CTACCTACAG 300

AGATTTAAAG CTCTAAGGTA AATATAAAAT TTTTAAGTGT ATAATGTGTT AAACTACTGA 360

TTCTAATTGT TTGTGTATTT TAGATTCCAA CCTATGGAAC TGATGAATGG GAGCAGTGGT 420

GGAATGCCTT TAATGAGGAA AACCTGTTTT GCTCAGAAGA AATGCCATCT AGTGATGATG 480

AGGCTACTGC TGACTCTCAA CATTCTACTC CTCCAAAAAA GAAGAGAAAG GTAGAAGACC 540

CCAAGGACTT TCCTTCAGAA TTGCTAAGTT TTTTGAGTCA TGCTGTGTTT AGTAATAGAA 600

CTCTTGCTTG CTTTGCTATT TACACCACAA AGGAAAAAGC TGCACTGCTA TACAAGAAAA 660

TTATGGAAAA ATATTCTGTA ACCTTTATAA GTAGGCATAA CAGTTATAAT CATAACATAC 720

TGTTTTTTCT TACTCCACAC AGGCATAGAG TGTCTGCTAT TAATAACTAT GCTCAAAAAT 780

TGTGTACCTT TAGCTTTTTA ATTTGTAAAG GGGTTAATAA GGAATATTTG ATGTATAGTG 840

CCTTGACTAG AGATCATAAT CAGCCATACC ACATTTGTAG AGGTTTTACT TGCTTTAAAA 900

AACCTCCCAC ACCTCCCCCT GAACCTGAAA CATAAAATGA ATGCAATTGT TGTTGTTAAC 960

TTGTTTATTG CAGCTTATAA TGGTTACAAA TAAAGCAATA GCATCACAAA TTTCACAAAT 1020

AAAGCATTTT TTTCACTGCA TTCTAGTTGT GGTTTGTCCA AACTCATCAA TGTATCTTAT 1080

CATGTCTGGA TCTGCCGGTC TCCCTATAGT GAGTCGTATT AATTTCGATA AGCCAGGTTA 1140

ACCTGCATTA ATGAATCGGC CAACGCGCGG GGAGAGGCGG TTTGCGTATT GGGCGCTCTT 1200

CCGCTTCCTC GCTCACTGAC TCGCTGCGCT CGGTCGTTCG GCTGCGGCGA GCGGTATCAG 1260

CTCACTCAAA GGCGGTAATA CGGTTATCCA CAGAATCAGG GGATAACGCA GGAAAGAACA 1320

TGTGAGCAAA AGGCCAGCAA AAGGCCAGGA ACCGTAAAAA GGACGCGTTG CTGGCGTTTT 1380

TCCATAGGCT CCGCCCCCCT GACGAGCATC ACAAAAATCG ACGCTCAAGT CAGAGGTGGC 1440

GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC TGGAAGCTCC CTCGTGCGCT 1500

CTCCTGTTCC GACCCTGCCG CTTACCGGAT ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG 1560

TGGCGCTTTC TCAATGCTCA CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA 1620

AGCTGGGCTG TGTGCACGAA CCCCCCGTTC AGCCCGACCG CTGCGCCTTA TCCGGTAACT 1680

ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC ACTGGCAGCA GCCACTGGTA 1740

ACAGGATTAG CAGAGCGAGG TATGTAGGCG GTGCTACAGA GTTCTTGAAG TGGTGGCCTA 1800

ACTACGGCTA CACTAGAAGG ACAGTATTTG GTATCTGCGC TCTGCTGAAG CCAGTTACCT 1860

TCGGAAAAAG AGTTGGTAGC TCTTGATCCG GCAAACAAAC CACCGCTGGT AGCGGTGGTT 1920

TTTTTGTTTG CAAGCAGCAG ATTACGCGCA GAAAAAAAGG ATCTCAAGAA GATCCTTTGA 1980

TCTTTTCTAC GGGGTCTGAC GCTCAGTGGA ACGAAAACTC ACGTTAAGGG ATTTTGGTCA 2040

TGAGATTATC AAAAAGGATC TTCACCTAGA TCCTTTTAAA TTAAAAATGA AGTTTTAAAT 2100

CAATCTAAAG TATATATGAG TAAACTTGGT CTGACAGTTA CCAATGCTTA ATCAGTGAGG 2160

CACCTATCTC AGCGATCTGT CTATTTCGTT CATCCATAGT TGCCTGACTC CCCGTCGTGT 2220

AGATAACTAC GATACGGGAG GGCTTACCAT CTGGCCCCAG TGCTGCAATG ATACCGCGAG 2280

ACCCACGCTC ACCGGCTCCA GATTTATCAG CAATAAACCA GCCAGCCGGA AGGGCCGAGC 2340

GCAGAAGTGG TCCTGCAACT TTATCCGCCT CCATCCAGTC TATTAATTGT TGCCGGGAAG 2400

CTAGAGTAAG TAGTTCGCCA GTTAATAGTT TGCGCAACGT TGTTGCCATT GCTACAGGCA 2460

TCGTGGTGTC ACGCTCGTCG TTTGGTATGG CTTCATTCAG CTCCGGTTCC CAACGATCAA 2520

GGCGAGTTAC ATGATCCCCC ATGTTGTGCA AAAAAGCGGT TAGCTCCTTC GGTCCTCCGA 2580

TCGTTGTCAG AAGTAAGTTG GCCGCAGTGT TATCACTCAT GGTTATGGCA GCACTGCATA 2640

ATTCTCTTAC TGTCATGCCA TCCGTAAGAT GCTTTTCTGT GACTGGTGAG TACTCAACCA 2700

AGTCATTCTG AGAATAGTGT ATGCGGCGAC CGAGTTGCTC TTGCCCGGCG TCAATACGGG 2760

ATAATACCGC GCCACATAGC AGAACTTTAA AAGTGCTCAT CATTGGAAAA CGTTCTTCGG 2820

GGCGAAAACT CTCAAGGATC TTACCGCTGT TGAGATCCAG TTCGATGTAA CCCACTCGTG 2880

CACCCAACTG ATCTTCAGCA TCTTTTACTT TCACCAGCGT TTCTGGGTGA GCAAAAACAG 2940

GAAGGCAAAA TGCCGCAAAA AAGGGAATAA GGGCGACACG GAAATGTTGA ATACTCATAC 3000

TCTTCCTTTT TCAATATTAT TGAAGCATTT ATCAGGGTTA TTGTCTCATG AGCGGATACA 3060

TATTTGAATG TATTTAGAAA AATAAACAAA TAGGGGTTCC GCGCACATTT CCCCGAAAAG 3120

TGCCACCTGA CGTCTAAGAA ACCATTATTA TCATGACATT AACCTATAAA AATAGGCGTA 3180

TCACGAGGCC CTTTCGTCTC GCGCGTTTCG GTGATGACGG TGAAAACCTC TGACACATGC 3240

AGCTCCCGGA GACGGTCACA GCTTGTCTGT AAGCGGATGC CGGGAGCAGA CAAGCCCGTC 3300

AGGGCGCGTC AGCGGGTGTT GGCGGGTGTC GGGGCTGGCT TAACTATGCG GCATCAGAGC 3360

AGATTGTACT GAGAGTGCAC CATATGGACA TATTGTCGTT AGAACGCGGC TACAATTAAT 3420

ACATAACCTT ATGTATCATA CACATACGAT TTAGGTGACA CTATAGAACT CGAGCAGAGC 3480

TTCCAAATTG AGAGAGAGGC TTAATCAGAG ACAGAAACTG TTTGAGTCAA CTCAAGGATG 3540

GTTTGAGGGA CTGTTTAACA GATCCCCTTG GTTTACCACC TTGATATCTA CCATTATGGG 3600

ACCCCTCATT GTACTCCTAA TGATTTTGCT CTTCGGACCC TGCATTCTTA ATCGATTAGT 3660

CCAATTTGTT AAAGACAGGA TATCAGTGGT CCAGGCTCTA GTTTTGACTC AACAATATCA 3720

CCAGCTGAAG CCTATAGAGT ACGAGCCATA GATAAAATAA AAGATTTTAT TTAGTCTCCA 3780

GAAAAAGGGG GGAATGAAAG ACCCCACCTG TAGGTTTGGC AAGCTAGCTT AAGTAACGCC 3840

ATTTTGCAAG GCATGGAAAA ATACATAACT GAGAATAGAG AAGTTCAGAT CAAGGTCAGG 3900

AACAGATGGA ACAGCTGAAT ATGGGCCAAA CAGGATATCT GTGGTAAGCA GTTCCTGCCC 3960

CGGCTCAGGG CCAAGAACAG ATGGAACAGC TGAATATGGG CCAAACAGGA TATCTGTGGT 4020

AAGCAGTTCC TGCCCCGGCT CAGGGCCAAG AACAGATGGT CCCCAGATGC GGTCCAGCCC 4080

TCAGCAGTTT CTAGAGAACC ATCAGATGTT TCCAGGGTGC CCCAAGGACC TGAAATGACC 4140

CTGTGCCTTA TTTGAACTAA CCAATCAGTT CGCTTCTCGC TTCTGTTCGC GCGCTTCTGC 4200

TCCCCGAGCT CAATAAAAGA GCCCACAACC CCTCACTCGG GGCGCCAGTC CTCCGATTGA 4260

CTGAGTCGCC CGG 4273

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 216 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CAAGAAAGCG GCTTCAGCTG TAAAGGACCT GGCCAGAATG TGGCTGTGAC CAGGGCACAC 60

CCTGACTCCC AAGGGAGGCG GCGGCGGCCT GAGCGGGGGG CCCGAGGAGG CCAGGTGTTT 120

TACAACAGCG AGTATGGGGA GCTGTCGGAG CCAAGCGAGG AGGACCACTG CTCCCCGTCT 180

GCCCGCGTGA CTTTCTTCAC AGACAACAGC TACTAA 216

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```
Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr Asn Thr Glu Ile Leu
1               5                  10                  15

Lys
```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1836 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 168..1412

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```
GCGGCCGCGT CGACGCAAAA GTTGCGAGCC GCCGAGTCCC GGGAGACGCT CGCCCAGGGG      60

GGTCCCCGGG AGGAAACCAC GGGACAGGGA CCAGGAGAGG ACCTCAGCCT CACGCCCCAG     120

CCTGCGCCAG CCAACGGACC GGCCTCCCTG CTCCCGGTCC ATCCACC ATG CAC TTG       176
                                                    Met His Leu
                                                      1

CTG TGC TTC TTG TCT CTG GCG TGT TCC CTG CTC GCC GCT GCG CTG ATC       224
Leu Cys Phe Leu Ser Leu Ala Cys Ser Leu Leu Ala Ala Ala Leu Ile
  5                  10                  15

CCC AGT CCG CGC GAG GCG CCC GCC ACC GTC GCC GCC TTC GAG TCG GGA       272
Pro Ser Pro Arg Glu Ala Pro Ala Thr Val Ala Ala Phe Glu Ser Gly
 20                  25                  30                  35

CTG GGC TTC TCG GAA GCG GAG CCC GAC GGG GGC GAG GTC AAG GCT TTT       320
Leu Gly Phe Ser Glu Ala Glu Pro Asp Gly Gly Glu Val Lys Ala Phe
                 40                  45                  50

GAA GGC AAA GAC CTG GAG GAG CAG TTG CGG TCT GTG TCC AGC GTA GAT       368
Glu Gly Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Ser Val Asp
             55                  60                  65

GAG CTG ATG TCT GTC CTG TAC CCA GAC TAC TGG AAA ATG TAC AAG TGC       416
Glu Leu Met Ser Val Leu Tyr Pro Asp Tyr Trp Lys Met Tyr Lys Cys
         70                  75                  80

CAG CTG CGG AAA GGC GGC TGG CAG CAG CCC ACC CTC AAT ACC AGG ACA       464
Gln Leu Arg Lys Gly Gly Trp Gln Gln Pro Thr Leu Asn Thr Arg Thr
  85                  90                  95

GGG GAC AGT GTA AAA TTT GCT GCT GCA CAT TAT AAC ACA GAG ATC CTG       512
Gly Asp Ser Val Lys Phe Ala Ala Ala His Tyr Asn Thr Glu Ile Leu
100                 105                 110                 115
```

```
AAA AGT ATT GAT AAT GAG TGG AGA AAG ACT CAA TGC ATG CCA CGT GAG        560
Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln Cys Met Pro Arg Glu
                120                 125                 130

GTG TGT ATA GAT GTG GGG AAG GAG TTT GGA GCA GCC ACA AAC ACC TTC        608
Val Cys Ile Asp Val Gly Lys Glu Phe Gly Ala Ala Thr Asn Thr Phe
        135                 140                 145

TTT AAA CCT CCA TGT GTG TCC GTC TAC AGA TGT GGG GGT TGC TGC AAC        656
Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys Gly Gly Cys Cys Asn
    150                 155                 160

AGG GAG GGG CTG CAG TGC ATG AAC ACC AGC ACA GGT TAC CTC AGC AAG        704
Arg Glu Gly Leu Gln Cys Met Asn Thr Ser Thr Gly Tyr Leu Ser Lys
165                 170                 175

ACG TTG TTT GAA ATT ACA GTG CCT CTC TCA CAA GGC CCC AAA CCA GTC        752
Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln Gly Pro Lys Pro Val
180                 185                 190                 195

ACA ATC AGT TTT GCC AAT CAC ACT TCC TGC CGG TGC ATG TCT AAA CTG        800
Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg Cys Met Ser Lys Leu
                200                 205                 210

GAT GTT TAC AGA CAA GTT CAT TCA ATT ATT AGA CGT TCT CTG CCA GCA        848
Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg Ser Leu Pro Ala
        215                 220                 225

ACA TTA CCA CAG TGT CAG GCA GCT AAC AAG ACA TGT CCA ACA AAC TAT        896
Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr Cys Pro Thr Asn Tyr
    230                 235                 240

GTG TGG AAT AAC TAC ATG TGC CGA TGC CTG GCT CAG CAG GAT TTT ATC        944
Val Trp Asn Asn Tyr Met Cys Arg Cys Leu Ala Gln Gln Asp Phe Ile
245                 250                 255

TTT TAT TCA AAT GTT GAA GAT GAC TCA ACC AAT GGA TTC CAT GAT GTC        992
Phe Tyr Ser Asn Val Glu Asp Asp Ser Thr Asn Gly Phe His Asp Val
260                 265                 270                 275

TGT GGA CCC AAC AAG GAG CTG GAT GAA GAC ACC TGT CAG TGT GTC TGC       1040
Cys Gly Pro Asn Lys Glu Leu Asp Glu Asp Thr Cys Gln Cys Val Cys
                280                 285                 290

AAG GGG GGG CTT CGG CCA TCT AGT TGT GGA CCC CAC AAA GAA CTA GAT       1088
Lys Gly Gly Leu Arg Pro Ser Ser Cys Gly Pro His Lys Glu Leu Asp
        295                 300                 305

AGA GAC TCA TGT CAG TGT GTC TGT AAA AAC AAA CTT TTC CCT AAT TCA       1136
Arg Asp Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe Pro Asn Ser
    310                 315                 320

TGT GGA GCC AAC AGG GAA TTT GAT GAG AAT ACA TGT CAG TGT GTA TGT       1184
Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys Val Cys
325                 330                 335

AAA AGA ACG TGT CCA AGA AAT CAG CCC CTG AAT CCT GGG AAA TGT GCC       1232
Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys Cys Ala
340                 345                 350                 355

TGT GAA TGT ACA GAA AAC ACA CAG AAG TGC TTC CTT AAA GGG AAG AAG       1280
Cys Glu Cys Thr Glu Asn Thr Gln Lys Cys Phe Leu Lys Gly Lys Lys
                360                 365                 370

TTC CAC CAT CAA ACA TGC AGT TGT TAC AGA AGA CCG TGT GCG AAT CGA       1328
Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys Ala Asn Arg
        375                 380                 385
```

```
CTG AAG CAT TGT GAT CCA GGA CTG TCC TTT AGT GAA GAA GTA TGC CGC      1376
Leu Lys His Cys Asp Pro Gly Leu Ser Phe Ser Glu Glu Val Cys Arg
        390                 395                 400

TGT GTC CCA TCG TAT TGG AAA AGG CCA CAT CTG AAC TAAGATCATA          1422
Cys Val Pro Ser Tyr Trp Lys Arg Pro His Leu Asn
    405                 410                 415

CCAGTTTTCA GTCAGTCACA GTCATTTACT CTCTTGAAGA CTGTTGGAAC AGCACTTAGC   1482

ACTGTCTATG CACAGAAAGA CTCTGTGGGA CCACATGGTA ACAGAGGCCC AAGTCTGTGT   1542

TTATTGAACC ATGTGGATTA CTGCGGGAGA GGACTGGCAC TCATGTGCAA AAAAAACCTC   1602

TTCAAAGACT GGTTTTCTGC CAGGGACCAG ACAGCTGAGG TTTTTCTCTT GTGATTTAAA   1662

AAAAGAATGA CTATATAATT TATTTCCACT AAAAATATTG TTCCTGCATT CATTTTTATA   1722

GCAATAACAA TTGGTAAAGC TCACTGTGAT CAGTATTTTT ATAACATGCA AAACTATGTT   1782

TAAAATAAAA TGAAAATTGT ATTATAAAAA AAAAAAAAAA AAAAAAAAAA GCTT         1836
```

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 415 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

```
Met His Leu Leu Cys Phe Leu Ser Leu Ala Cys Ser Leu Leu Ala Ala
 1               5                  10                  15

Ala Leu Ile Pro Ser Pro Arg Glu Ala Pro Ala Thr Val Ala Ala Phe
            20                  25                  30

Glu Ser Gly Leu Gly Phe Ser Glu Ala Glu Pro Asp Gly Gly Glu Val
        35                  40                  45

Lys Ala Phe Glu Gly Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser
    50                  55                  60

Ser Val Asp Glu Leu Met Ser Val Leu Tyr Pro Asp Tyr Trp Lys Met
65                  70                  75                  80

Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln Gln Pro Thr Leu Asn
                85                  90                  95

Thr Arg Thr Gly Asp Ser Val Lys Phe Ala Ala Ala His Tyr Asn Thr
            100                 105                 110

Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln Cys Met
        115                 120                 125

Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Ala Ala Thr
    130                 135                 140

Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys Gly Gly
145                 150                 155                 160

Cys Cys Asn Arg Glu Gly Leu Gln Cys Met Asn Thr Ser Thr Gly Tyr
                165                 170                 175

Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln Gly Pro
            180                 185                 190
```

Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg Cys Met
195 200 205

Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg Ser
210 215 220

Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr Cys Pro
225 230 235 240

Thr Asn Tyr Val Trp Asn Asn Tyr Met Cys Arg Cys Leu Ala Gln Gln
245 250 255

Asp Phe Ile Phe Tyr Ser Asn Val Glu Asp Asp Ser Thr Asn Gly Phe
260 265 270

His Asp Val Cys Gly Pro Asn Lys Glu Leu Asp Glu Asp Thr Cys Gln
275 280 285

Cys Val Cys Lys Gly Gly Leu Arg Pro Ser Ser Cys Gly Pro His Lys
290 295 300

Glu Leu Asp Arg Asp Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe
305 310 315 320

Pro Asn Ser Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln
325 330 335

Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly
340 345 350

Lys Cys Ala Cys Glu Cys Thr Glu Asn Thr Gln Lys Cys Phe Leu Lys
355 360 365

Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys
370 375 380

Ala Asn Arg Leu Lys His Cys Asp Pro Gly Leu Ser Phe Ser Glu Glu
385 390 395 400

Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro His Leu Asn
405 410 415

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TCTCTTCTGT GCTTGAGTTG AG 22

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

TCTCTTCTGT CCCTGAGTTG AG 22

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TGTGCTGCAG CAAATTTTAT AGTCTCTTCT GTGGCGGCGG CGGCGGCGGG CGCCTCGCGA 60

GGACC 65

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CTGGCAGGGA ACTGCTAATA ATGGAATGAA 30

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 84 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GGGCTCCGCG TCCGAGAGGT CGAGTCCGGA CTCGTGATGG TGATGGTGAT GGGCGGCGGC 60

GGCGGCGGGC GCCTCGCGAG GACC 84

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GTATTATAAT GTCCTCCACC AAATTTTATA G 31

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 93 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GTTCGCTGCC TGACACTGTG GTAGTGTTGC TGGCGGCCGC TAGTGATGGT GATGGTGATG 60

AATAATGGAA TGAACTTGTC TGTAAACATC CAG 93

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CATGTACGAA CCGCCAGG 18

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

AATGACCAGA GAGAGGCGAG 20

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Ala Val Val Met Thr Gln Thr Pro Ala Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1741 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 453..1706

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

GCCCCCGCCG AGCGCTCCGC GCGCAGCCGC CGGGCCGGGC CGGCCCGCGG AGGGCGCGCT 60

GCGAGCGGCC ACTGGGTCCT GCTTCCCTCC TTCCTCTCCC TCCTCCTCCT CCTCCTTCTC 120

TCTGCGCTTT CCACCGCTCC CGAGCGAGCG CACGCTCGGA TGTCCGGTTT CCTGGTGGGT 180

```
TTTTTACCTG GCAAAGTCCG GATAACTTCG GTGAGAATTT GCAAAGAGGC TGGGAGCTCC      240

CCTGCAGGCG TCTGGGAGCT GCTGCCGCCG TCGCATCTTC TCCATCCCGC GGATTTTACT      300

GCCTTGGATA TTGCGAGGGG AGGGAGGGGG GTGAGGACAG CAAAAAGAAA GGGGTGGGGG      360

GGGGGAGAGA AAAGGAAAAG AAGGAGCCTC GGAATTGTGC CCGCATTCCT GCGCTGCCCC      420

GCGGCCCCCC TCCGCTCTGC CATCTCCGCA CA ATG CAC TTG CTG GAG ATG CTC         473
                                    Met His Leu Leu Glu Met Leu
                                      1               5

TCC CTG GGC TGC TGC CTC GCT GCT GGC GCC GTG CTC CTG GGA CCC CGG         521
Ser Leu Gly Cys Cys Leu Ala Ala Gly Ala Val Leu Leu Gly Pro Arg
         10                  15                  20

CAG CCG CCC GTC GCC GCC GCC TAC GAG TCC GGG CAC GGC TAC TAC GAG         569
Gln Pro Pro Val Ala Ala Ala Tyr Glu Ser Gly His Gly Tyr Tyr Glu
     25                  30                  35

GAG GAG CCC GGT GCC GGG GAA CCC AAG GCT CAT GCA AGC AAA GAC CTG         617
Glu Glu Pro Gly Ala Gly Glu Pro Lys Ala His Ala Ser Lys Asp Leu
 40                  45                  50                  55

GAA GAG CAG TTG CGA TCT GTG TCC AGT GTG GAT GAA CTC ATG ACA GTA         665
Glu Glu Gln Leu Arg Ser Val Ser Ser Val Asp Glu Leu Met Thr Val
                 60                  65                  70

CTT TAC CCA GAA TAC TGG AAA ATG TTC AAA TGT CAG TTG AGG AAA GGA         713
Leu Tyr Pro Glu Tyr Trp Lys Met Phe Lys Cys Gln Leu Arg Lys Gly
             75                  80                  85

GGT TGG CAA CAC AAC AGG GAA CAC TCC AGC TCT GAT ACA AGA TCA GAT         761
Gly Trp Gln His Asn Arg Glu His Ser Ser Ser Asp Thr Arg Ser Asp
         90                  95                  100

GAT TCA TTG AAA TTT GCC GCA GCA CAT TAT AAT GCA GAG ATC CTG AAA         809
Asp Ser Leu Lys Phe Ala Ala Ala His Tyr Asn Ala Glu Ile Leu Lys
     105                 110                 115

AGT ATT GAT ACT GAA TGG AGA AAA ACC CAG GGC ATG CCA CGT GAA GTG         857
Ser Ile Asp Thr Glu Trp Arg Lys Thr Gln Gly Met Pro Arg Glu Val
120                 125                 130                 135

TGT GTG GAT TTG GGG AAA GAG TTT GGA GCA ACT ACA AAC ACC TTC TTT         905
Cys Val Asp Leu Gly Lys Glu Phe Gly Ala Thr Thr Asn Thr Phe Phe
                140                 145                 150

AAA CCC CCG TGT GTA TCC ATC TAC AGA TGT GGA GGT TGC TGC AAT AGT         953
Lys Pro Pro Cys Val Ser Ile Tyr Arg Cys Gly Gly Cys Cys Asn Ser
            155                 160                 165

GAA GGA CTC CAG TGT ATG AAT ATC AGC ACA AAT TAC ATC AGC AAG ACA        1001
Glu Gly Leu Gln Cys Met Asn Ile Ser Thr Asn Tyr Ile Ser Lys Thr
        170                 175                 180

TTG TTT GAG ATT ACA GTG CCT CTC TCT CAT GGC CCC AAA CCT GTA ACA        1049
Leu Phe Glu Ile Thr Val Pro Leu Ser His Gly Pro Lys Pro Val Thr
    185                 190                 195

GTC AGT TTT GCC AAT CAC ACG TCC TGC CGA TGC ATG TCT AAG TTG GAT        1097
Val Ser Phe Ala Asn His Thr Ser Cys Arg Cys Met Ser Lys Leu Asp
200                 205                 210                 215

GTT TAC AGA CAA GTT CAT TCT ATC ATA AGA CGT TCC TTG CCA GCA ACA        1145
Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg Ser Leu Pro Ala Thr
                220                 225                 230
```

```
CAA ACT CAG TGT CAT GTG GCA AAC AAG ACC TGT CCA AAA AAT CAT GTC      1193
Gln Thr Gln Cys His Val Ala Asn Lys Thr Cys Pro Lys Asn His Val
            235                 240                 245

TGG AAT AAT CAG ATT TGC AGA TGC TTA GCA CAG CAC GAT TTT GGT TTC      1241
Trp Asn Asn Gln Ile Cys Arg Cys Leu Ala Gln His Asp Phe Gly Phe
        250                 255                 260

TCT TCT CAC CTT GGA GAT TCT GAC ACA TCT GAA GGA TTC CAT ATT TGT      1289
Ser Ser His Leu Gly Asp Ser Asp Thr Ser Glu Gly Phe His Ile Cys
    265                 270                 275

GGG CCC AAC AAA GAG CTG GAT GAA GAA ACC TGT CAA TGC GTC TGC AAA      1337
Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys Val Cys Lys
280                 285                 290                 295

GGA GGT GTG CGG CCC ATA AGC TGT GGC CCT CAC AAA GAA CTA GAC AGG      1385
Gly Gly Val Arg Pro Ile Ser Cys Gly Pro His Lys Glu Leu Asp Arg
                300                 305                 310

GCA TCA TGT CAG TGC ATG TGC AAA AAC AAA CTG CTC CCC AGT TCC TGT      1433
Ala Ser Cys Gln Cys Met Cys Lys Asn Lys Leu Leu Pro Ser Ser Cys
            315                 320                 325

GGG CCT AAC AAA GAA TTT GAT GAA GAA AAG TGC CAG TGT GTA TGT AAA      1481
Gly Pro Asn Lys Glu Phe Asp Glu Glu Lys Cys Gln Cys Val Cys Lys
        330                 335                 340

AAG ACC TGT CCC AAA CAT CAT CCA CTA AAT CCT GCA AAA TGC ATC TGC      1529
Lys Thr Cys Pro Lys His His Pro Leu Asn Pro Ala Lys Cys Ile Cys
    345                 350                 355

GAA TGT ACA GAA TCT CCC AAT AAA TGT TTC TTA AAA GGA AAA AGA TTT      1577
Glu Cys Thr Glu Ser Pro Asn Lys Cys Phe Leu Lys Gly Lys Arg Phe
360                 365                 370                 375

CAT CAC CAG ACA TGC AGT TGT TAC AGA CCA CCA TGT ACA GTC CGA ACG      1625
His His Gln Thr Cys Ser Cys Tyr Arg Pro Pro Cys Thr Val Arg Thr
                380                 385                 390

AAA CGC TGT GAT GCT GGA TTT CTG TTA GCT GAA GAA GTG TGC CGC TGT      1673
Lys Arg Cys Asp Ala Gly Phe Leu Leu Ala Glu Glu Val Cys Arg Cys
            395                 400                 405

GTA CGC ACA TCT TGG AAA AGA CCA CTT ATG AAT TAAGCGAAGA AAGCACTACT    1726
Val Arg Thr Ser Trp Lys Arg Pro Leu Met Asn
        410                 415

CGCTATATAG TGTCG                                                    1741
```

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 418 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

```
Met His Leu Leu Glu Met Leu Ser Leu Gly Cys Cys Leu Ala Ala Gly
  1               5                  10                  15

Ala Val Leu Leu Gly Pro Arg Gln Pro Pro Val Ala Ala Ala Tyr Glu
            20                  25                  30

Ser Gly His Gly Tyr Tyr Glu Glu Glu Pro Gly Ala Gly Glu Pro Lys
        35                  40                  45
```

```
Ala His Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Ser
     50                  55                  60

Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Phe
 65                  70                  75                  80

Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu His Ser
                 85                  90                  95

Ser Ser Asp Thr Arg Ser Asp Asp Ser Leu Lys Phe Ala Ala Ala His
            100                 105                 110

Tyr Asn Ala Glu Ile Leu Lys Ser Ile Asp Thr Glu Trp Arg Lys Thr
        115                 120                 125

Gln Gly Met Pro Arg Glu Val Cys Val Asp Leu Gly Lys Glu Phe Gly
    130                 135                 140

Ala Thr Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Ile Tyr Arg
145                 150                 155                 160

Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Ile Ser
                165                 170                 175

Thr Asn Tyr Ile Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser
            180                 185                 190

His Gly Pro Lys Pro Val Thr Val Ser Phe Ala Asn His Thr Ser Cys
        195                 200                 205

Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile
    210                 215                 220

Arg Arg Ser Leu Pro Ala Thr Gln Thr Gln Cys His Val Ala Asn Lys
225                 230                 235                 240

Thr Cys Pro Lys Asn His Val Trp Asn Asn Gln Ile Cys Arg Cys Leu
                245                 250                 255

Ala Gln His Asp Phe Gly Phe Ser Ser His Leu Gly Asp Ser Asp Thr
            260                 265                 270

Ser Glu Gly Phe His Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu
        275                 280                 285

Thr Cys Gln Cys Val Cys Lys Gly Gly Val Arg Pro Ile Ser Cys Gly
    290                 295                 300

Pro His Lys Glu Leu Asp Arg Ala Ser Cys Gln Cys Met Cys Lys Asn
305                 310                 315                 320

Lys Leu Leu Pro Ser Ser Cys Gly Pro Asn Lys Glu Phe Asp Glu Glu
                325                 330                 335

Lys Cys Gln Cys Val Cys Lys Lys Thr Cys Pro Lys His His Pro Leu
            340                 345                 350

Asn Pro Ala Lys Cys Ile Cys Glu Cys Thr Glu Ser Pro Asn Lys Cys
        355                 360                 365

Phe Leu Lys Gly Lys Arg Phe His His Gln Thr Cys Ser Cys Tyr Arg
    370                 375                 380

Pro Pro Cys Thr Val Arg Thr Lys Arg Cys Asp Ala Gly Phe Leu Leu
385                 390                 395                 400

Ala Glu Glu Val Cys Arg Cys Val Arg Thr Ser Trp Lys Arg Pro Leu
                405                 410                 415

Met Asn
```

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1582 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CTAGAGTTGA ACCAGATAAG AAAGTCTCTT CTTCCGGTAA GATATTATGG ACCTATAACA 60

TCTGTGTACT TAAAAGTAGA TTGGGAGTGA AAGGCAGACT TTTGATGTTC TGTACACTGT 120

TGAAACCCCT TAGCGTGGTC CTCTGTAACC TGCTCACCCT GCCCCAAGGA GGCAGCTAGC 180

CAATGCCACC AGCCCAACGG AAACCCCAGT GCTTTTCCAA TGGGGAAATG CAGTCACTTT 240

TCTTTGGATG CTACACATCC TTTCTGGAAT ATGTCTCACA CACATCTCTC TTTATCACCC 300

CCTTTTTCAA GTAAACCAAC TTCTTGCAGA AGCTGACAAT GTGTCTCTTT ACTCTCCACG 360

AAGATTCTGG CCCTTCTCTT CACCTGTCAG AAGTTTAGGA TTCCAAAGGG ATCATTAGCA 420

TCCATCCCAA CAGCCTGCAC TGCATCCTGA GAACTGCGGT TCTTGGATCA TCAGGCAACT 480

TTCAACTACA CAGACCAAGG GAGAGAGGGG ACCCCTCCGA GGTCCCATAG GGTTCTCTGA 540

CATAGTGATG ACCTTTTTCC AAACTTTGAG CAGGGCGCTG GGGGCCAGGC GTGCGGGAGG 600

GAGGACAAGA ACTCGGGAGT GGCCGAGGAT AAAGCGGGGG CTCCCTCCAC CCCACGGTGC 660

CCAGTTTCTC CCCGCTGCAC GTGGTCCAGG GTGGTCGCAT CACCTCTAAA GCCGGTCCCG 720

CCAACCGCCA GCCCCGGGAC TGAACTTGCC CCTCCGGCCG CCCGCTCCCC GCAGGGGACA 780

GGGGCGGGGA GGGAGAGATC CAGAGGGGGG CTGGGGGAGG TGGGNCCGCC GGGGAGGAGN 840

CGAGGGAAAC GGGGAGCTCC AGGGAGACGG CTTCCGAGGG AGAGTGAGAG GGGAGGGCAG 900

CCCGGGCTCG GCACGCTCCC TCCCTCGGCC GCTTTCTCTC ACATAAGCGC AGGCAGAGGG 960

CGCGTCAGTC ATGCCCTGCC CCTGCGCCCG CCGCCGCCGC CGCCGCCGCT CAGCCCGGCG 1020

CGCTCTGGAG GATCCTGCGC CGCGGCGCTC CCGGGCCCCG CCGCCGCCAG CCGCCCCGGC 1080

GGCCCTCCTC CCGCCCCCGG CACCGCCGCC AGCGCCCCCG CCGCAGCGCC CGCGGCCCGG 1140

CTCCTCTCAC TTCGGGGAAG GGGAGGGAGG AGGGGGACGA GGGCTCTGGC GGGTTTGGAG 1200

GGGCTGAACA TCGCGGGGTG TTCTGGTGTC CCCCGCCCCG CCTCTCCAAA AAGCTACACC 1260

GACGCGGACC GCGGCGGCGT CCTCCCTCGC CCTCGCTTCA CCTCGCGGGC TCCGAATGCG 1320

GGGAGCTCGG ATGTCCGGTT TCCTGTGAGG CTTTTACCTG ACACCCGCCG CCTTTCCCCG 1380

GCACTGGCTG GGAGGGCGCC CTGCAAAGTT GGGAACGCGG AGCCCCGGAC CCGCTCCCGC 1440

CGCCTCCGGC TCGCCCAGGG GGGGTCGCCG GGAGGAGCCC GGGGGAGAGG GACCAGGAGG 1500

GGCCCGCGGC CTCGCAGGGG CGCCCGCGCC CCCACCCCTG CCCCCGCCAG CGGACCGGTC 1560

CCCCACCCCC GGTCCTTCCA CC 1582

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CACGGCTTAT GCAAGCAAAG 20

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

AACACAGTTT TCCATAATAG 20

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

GCCACGGTAG GTCTGCGT 18

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

TTTCTTTGAC AGGCTTAT 18

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

ATCTTGAAAA GTAAGTATGG G 21

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

ATGACTTGAC AGGTATTGAT 20

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

AGCAAGACGG TGGGTATTGT 20

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

CCCTTCTTTG TAGTTATTTG AA 22

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

CCACAGTGAG TATGAATTAA 20

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

TTCTTCCAAA GGTGTCAG 18

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

GGAGATGGTA GCAGAATG 18

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

CTATTTGTCT AGACTCAACA GAT 23

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

CAAACATGCA GGTAAGAGAT CC 22

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

TGTTCTCCTA GCTGTTACAG A 21

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

GGCGAGGTCA AGGTAGGTGC AAGG 24

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

ATTGTCTTTG ACAGGCTTTT TGAAGG 26

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

GAGATCCTGA AAAGTAAGTA G 21

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

TGTGACTCGA CAGGTATTGA TAAT 24

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

CTCAGCAAGA CGGTAGGTAT 20

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

TTGTCCCTTG TAGTTGTTTG AAATT 25

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

ACATTACCAC AGTGAGTATG 20

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

GTCTCCCCAA AAGGTGTCAG GCAGCT 26

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

AATGTTGAAG ATGGTAAGTA AAA 23

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

TCTAGACTCA ACCAAT 16

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

CAAACATGCA GGTAAGGAGT GT 22

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TTTTCCCCTA GTTGTTACAG AAGA 24

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Leu Ser Lys Thr Val Ser Gly Ser Glu Gln Asp Leu Pro His Glu Leu
1 5 10 15

His Val Glu

CLAIMS

1. A purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase.

2. A mammalian polypeptide according to claim 1.

3. A human polypeptide according to claim 1.

4. A polypeptide according to claim 3 having an apparent molecular weight of about 32 kD as assessed by SDS-PAGE under reducing conditions.

5. A polypeptide according to claim 3 that is substantially free of other human polypeptides.

6. A purified and isolated polypeptide according to claim 1, said polypeptide being encoded by plasmid pFLT4-L, deposited as ATCC Accession Number 97231.

7. A polypeptide according to claim 1 having an amino acid sequence comprising a portion of SEQ ID NO: 33.

8. A polypeptide according to claim 1 comprising an amino acid sequence set forth in SEQ ID NO: 33 from about residue 161 of SEQ ID NO: 33 to about residue 211 of SEQ ID NO: 33.

9. A polypeptide according to claim 1 comprising an amino acid sequence set forth in SEQ ID NO: 33 from about residue 131 of SEQ ID NO: 33 to about residue 211 of SEQ ID NO: 33.

10. A polypeptide according to claim 1 comprising an amino acid sequence set forth in SEQ ID NO: 33 from residue 113 of SEQ ID NO: 33 to residue 213 of SEQ ID NO: 33.

11. A polypeptide according to claim 1 comprising an amino acid sequence set forth in SEQ ID NO: 33 from residue 113 of SEQ ID NO: 33 to residue 227 of SEQ ID NO: 33.

12. A polypeptide according to claim 1 comprising amino acids 103 to 217 of SEQ ID NO: 33.

13. A polypeptide according to claim 1 comprising amino acids 103 to 225 of SEQ ID NO: 33.

14. A polypeptide according to claim 1 comprising amino acids 103 to 227 of SEQ ID NO: 33.

15. A polypeptide according to claim 1 comprising amino acids 32 to 227 of SEQ ID NO: 33.

16. A polypeptide according to any of claims 1-15, wherein said polypeptide is capable of stimulating tyrosine phosphorylation of Flt4 receptor tyrosine kinase in a host cell expressing said Flt4 receptor tyrosine kinase.

17. A polypeptide according to claim 1 having the amino acid sequence of residues 1 to 419 of SEQ ID NO: 33.

18. A polypeptide according to any of claims 1-17 further comprising a detectable label.

19. A purified protein comprising a first polypeptide linked to a second polypeptide, wherein at least one of said first polypeptide and said second polypeptide is a polypeptide according to any of claims 1-17, and wherein said protein is capable of binding to Flt4 receptor tyrosine kinase.

20. A purified protein according to claim 19 wherein said first polypeptide is covalently linked to said

second polypeptide.

21. A purified protein according to claim 19 wherein each of said first polypeptide and said second polypeptide is a polypeptide according to any of claims 1-17.

22. A purified and isolated nucleic acid comprising a nucleotide sequence encoding a polypeptide according to any of claims 1-17.

23. A nucleic acid according to claim 22 comprising a nucleotide sequence encoding a polypeptide consisting of amino acids 113 to 213 of SEQ ID NO: 33.

24. A nucleic acid according to claim 22 comprising a nucleotide sequence encoding a polypeptide consisting of amino acids 103 to 217 of SEQ ID NO: 33.

25. A nucleic acid according to claim 22 having the nucleotide sequence of nucleotides 352 to 1608 of SEQ ID NO: 32.

26. A nucleic acid according to claim 22 comprising a VEGF-C encoding insert of plasmid pFLT4-L, deposited as ATCC Accession Number 97321.

27. A vector comprising a nucleic acid according to any of claims 22-26.

28. A host cell transformed or transfected with a nucleic acid according to any of claims 22-26.

29. An antibody which is specifically reactive with a polypeptide according to any of claims 1-17.

30. An antibody according to claim 29 which is a monoclonal antibody.

31. A pharmaceutical composition comprising a polypeptide according to any of claims 1-17 in a pharmaceutically acceptable diluent, adjuvant, excipient, or carrier.

32. A method of making a polypeptide capable of specifically binding to Flt4 receptor tyrosine kinase, said method comprising the steps of:

(a) expressing a nucleic acid according to any of claims 22-26 in a host cell; and

(b) purifying a polypeptide capable of specifically binding to Flt4 receptor tyrosine kinase from said host cell or from a growth medium of said host cell.

33. A polypeptide capable of specifically binding to Flt4 receptor tyrosine kinase, said polypeptide produced by the method according to claim 32.

34. A murine polypeptide according to claim 1.

35. A polypeptide according to claim 34, said polypeptide comprising a portion of the amino acid sequence set forth in SEQ ID NO: 41, said portion being capable of specifically binding to an Flt4 receptor tyrosine kinase.

36. A purified and isolated nucleic acid encoding the polypeptide according to claim 34 or 35.

37. A purified and isolated nucleic acid having at least about 16 nucleotides, said nucleic acid specifically hybridizing to a human gene encoding VEGF-C.

38. A nucleic acid according to claim 37 which hybridizes to a human gene encoding VEGF-C, under hybridization conditions wherein said nucleic acid fails to hybridize to a human gene encoding VEGF or VEGF-B, and wherein said nucleic acid comprises a continuous nucleotide sequence of at least twenty nucleotides from a nucleotide sequence selected from the group consisting of:

SEQ ID NO: 32, and

a nucleotide sequence complementary to SEQ ID NO: 32.

39. A method for detecting endothelial cells in a biological tissue comprising the steps of:

exposing a biological tissue comprising endothelial cells to a polypeptide according to any of claims 1-18, under conditions wherein said polypeptide binds to endothelial cells; and

detecting said polypeptide bound to endothelial cells in said biological tissue, thereby detecting said endothelial cells.

40. The method according to claim 39, further comprising the step of washing said biological tissue, said washing step being performed after said exposing step and before said detecting step.

41. A method of modulating the growth of mammalian endothelial cells comprising the steps of:

exposing mammalian endothelial cells to a polypeptide according to any of claims 1-17 in an amount effective to modulate the growth of mammalian endothelial cells.

42. A purified and isolated polypeptide which is capable of binding to Kdr (VEGFR-2), said polypeptide having an amino acid sequence comprising a portion of SEQ ID NO: 33.

43. A purified nucleic acid comprising a promoter for VEGF-C.

44. A nucleic acid according to claim 43 comprising a portion of SEQ ID NO: 54, wherein said portion is capable of promoting expression of a protein encoding gene operatively linked thereto under conditions wherein VEGF-C is expressed in native host cells.

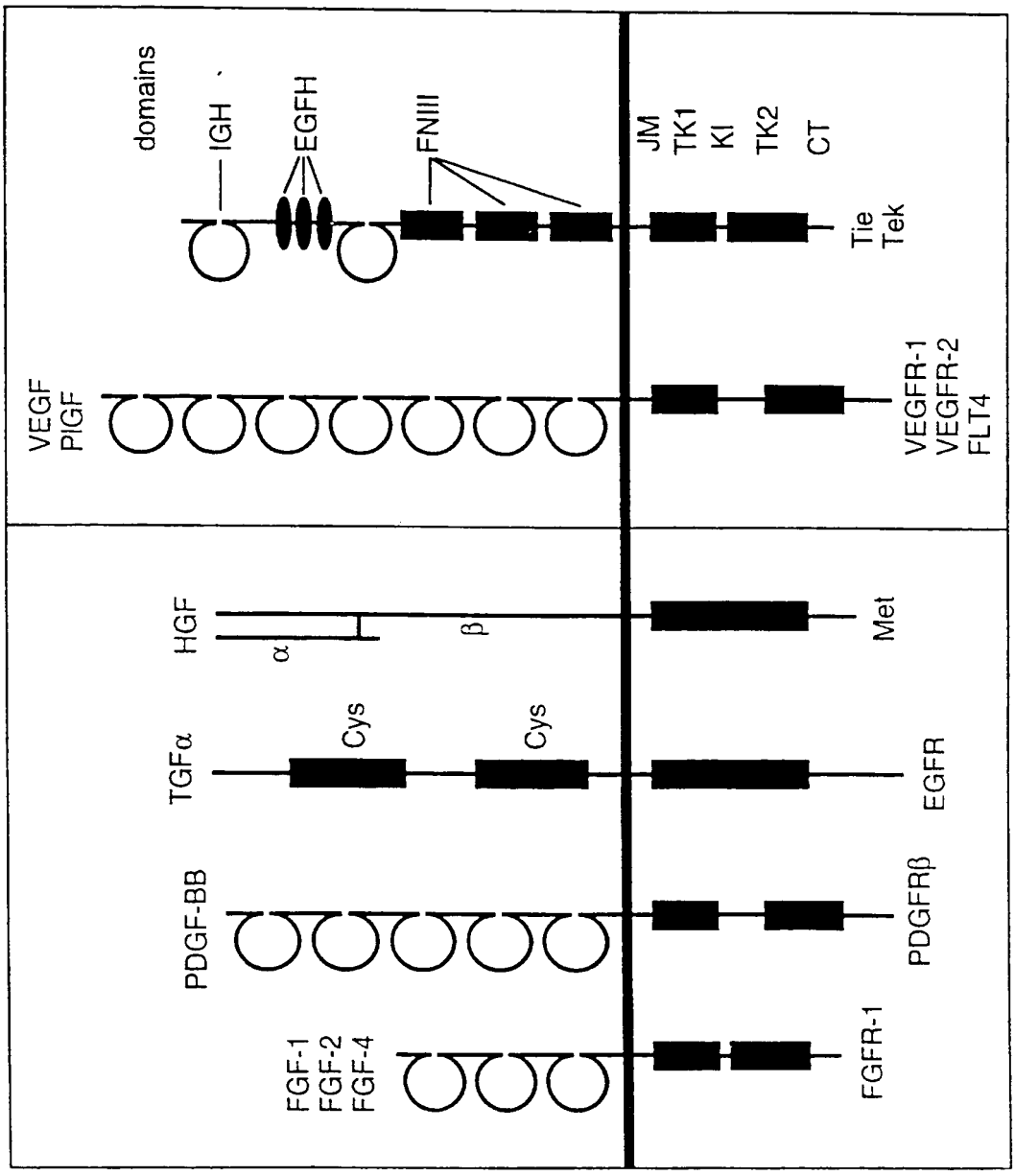

domains
IGH
EGFH
FNIII
JM
TK1
KI
TK2
CT
Tie
Tek
VEGF
PlGF
VEGFR-1
VEGFR-2
FLT4
HGF
α
β
Met
TGFα
Cys
Cys
EGFR
PDGF-BB
PDGFRβ
FGF-1
FGF-2
FGF-4
FGFR-1

Figure 1

Figure 2 (1 of 2)

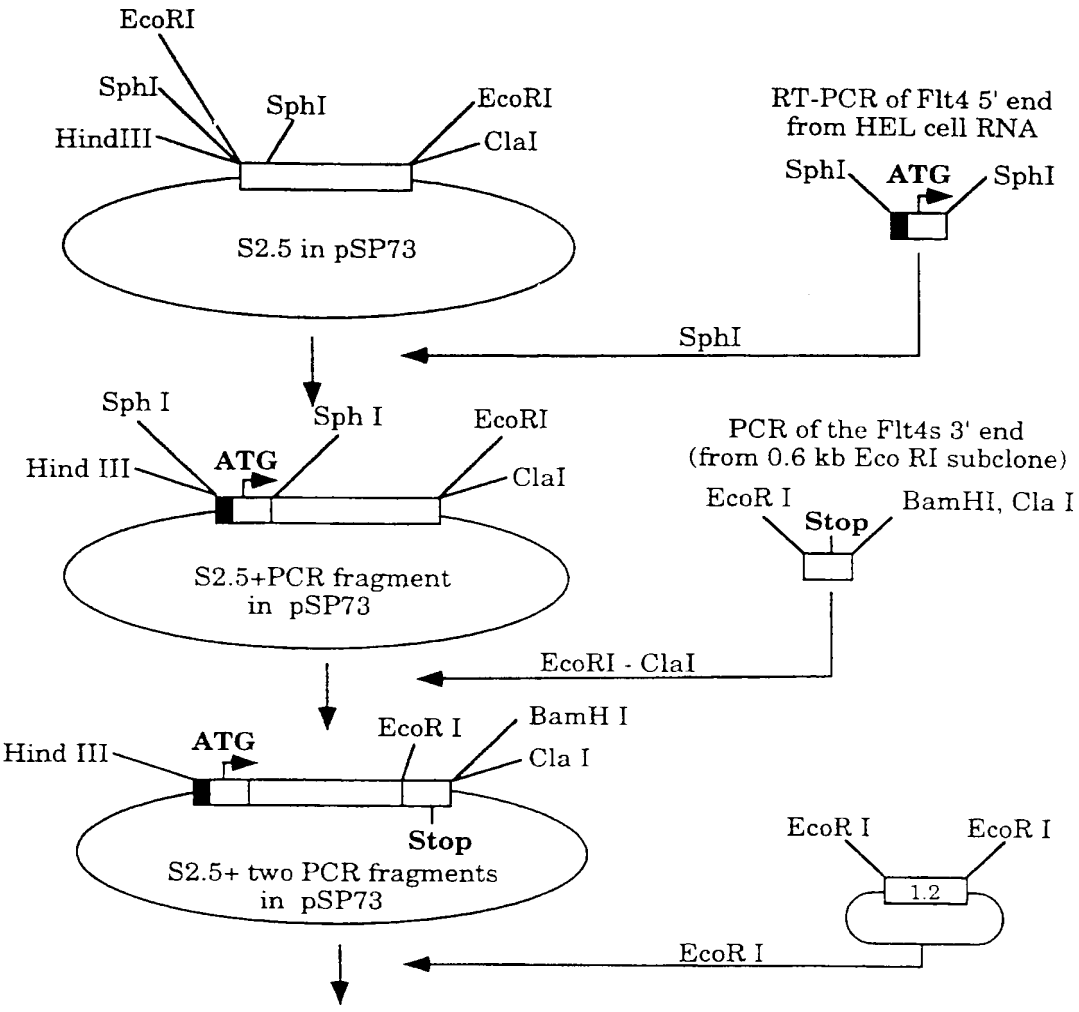


Figure 2 (2 of 2)

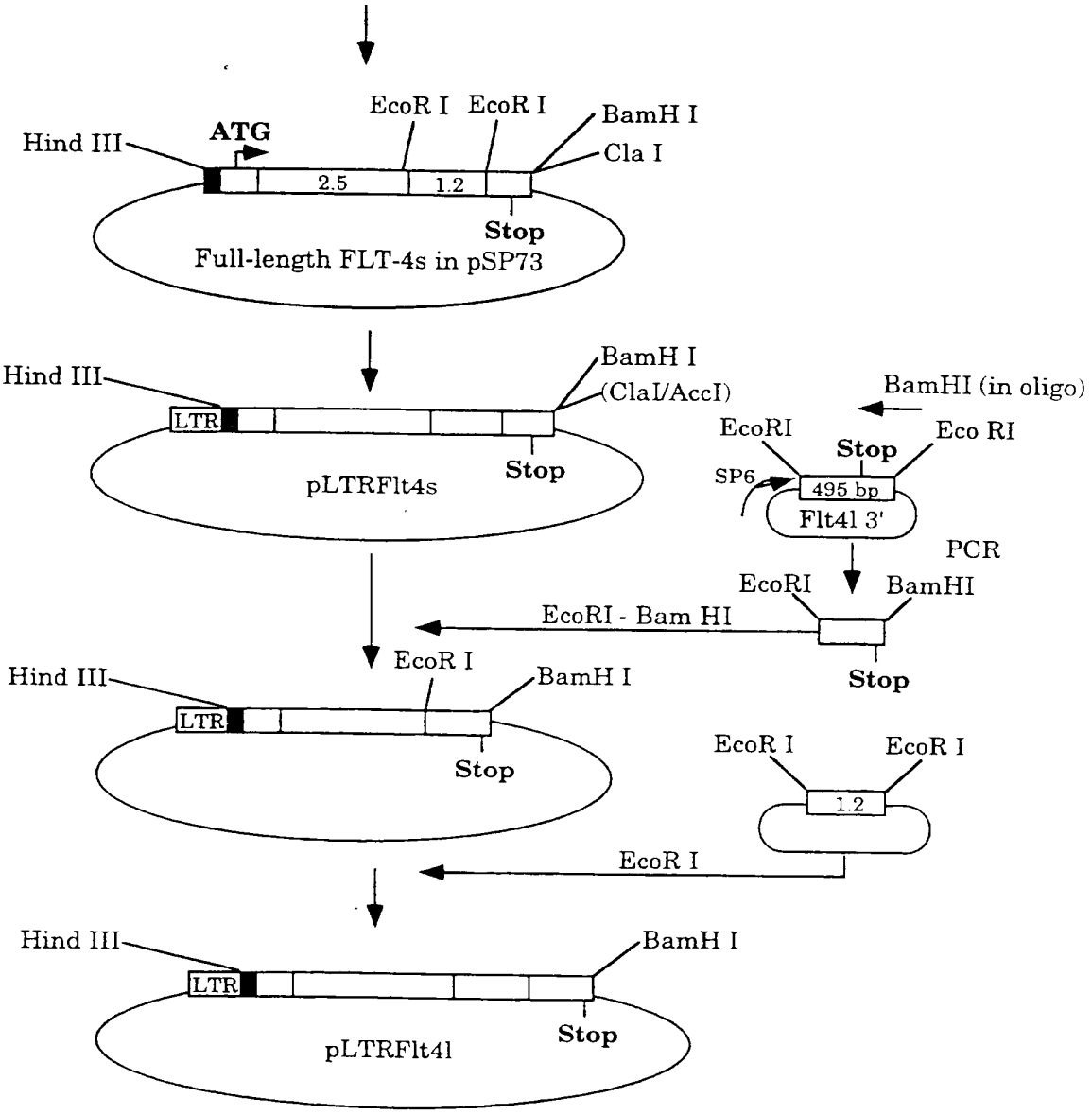
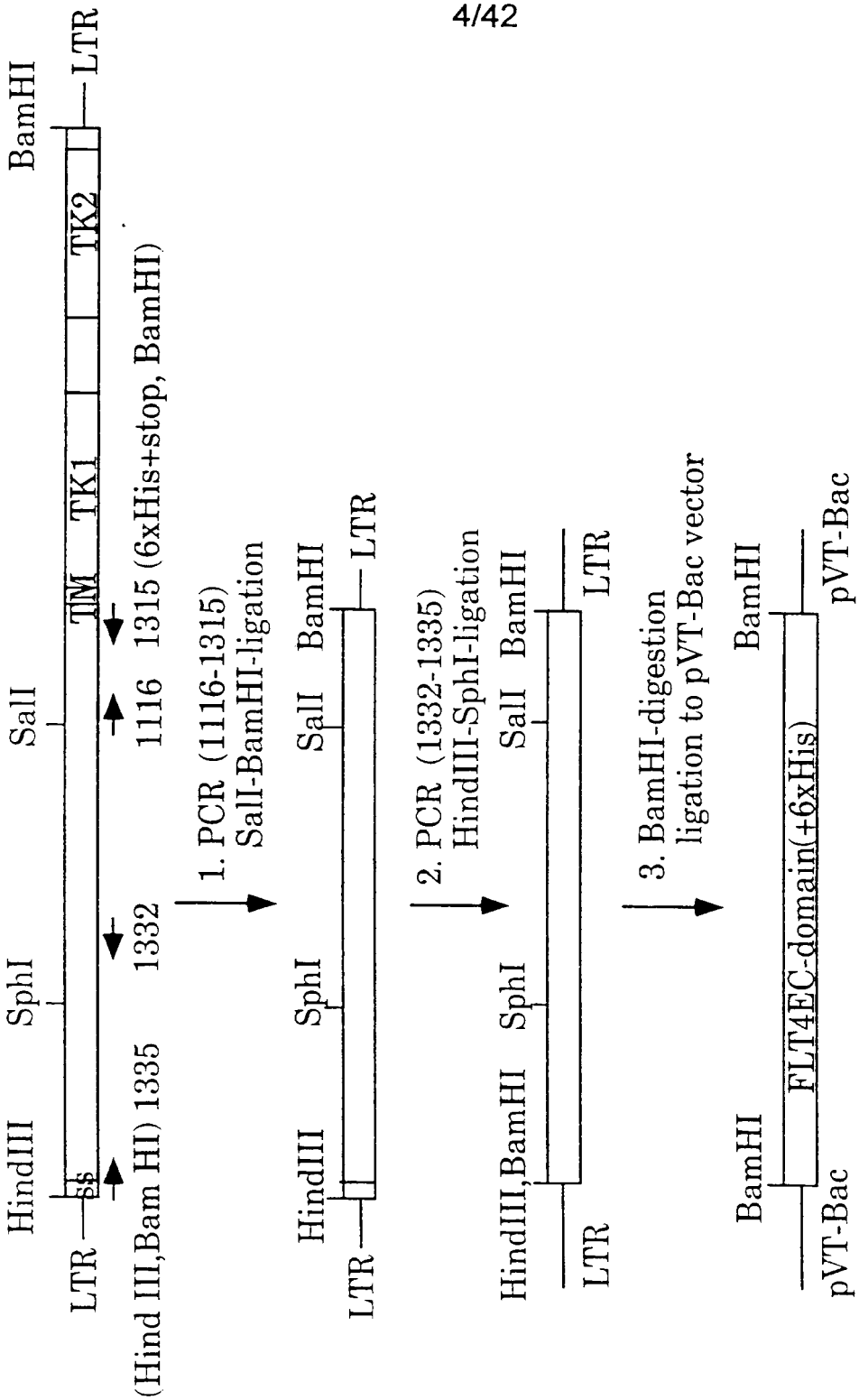


Figure 3


HindIII
SphI
SalI
BamHI
LTR
TM
TK1
TK2
(Hind III,Bam HI) 1335
1332
1116
1315 (6xHis+stop, BamHI)
1. PCR (1116-1315)
SalI-BamHI-ligation
2. PCR (1332-1335)
HindIII-SphI-ligation
3. BamHI-digestion
ligation to pVT-Bac vector
HindIII,BamHI
FLT4EC-domain(+6xHis)
pVT-Bac

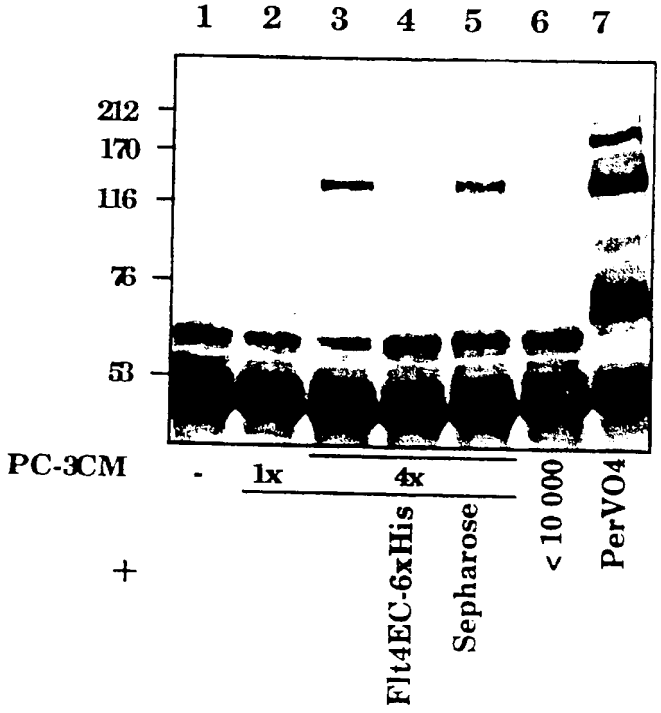

1 2 3 4 5 6 7
212
170
116
76
53
anti-PTyr
PC-3CM
-
+
1x
4x
Flt4EC-6xHis
Sepharose
< 10 000
PerVO4

Figure 4

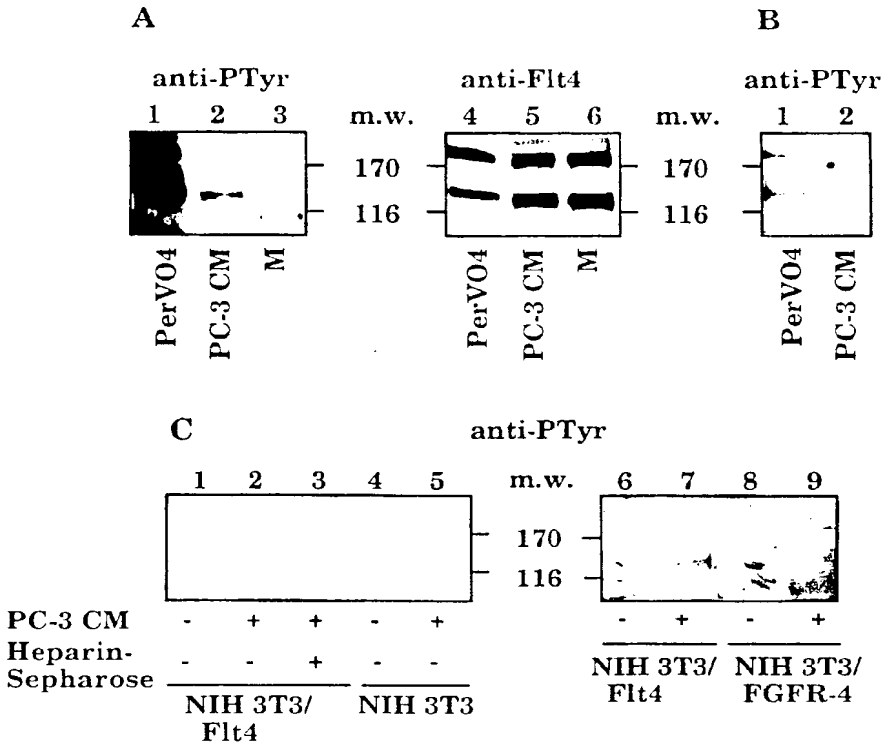

A
anti-PTyr
1 2 3
m.w.
170
116
PerVO4
PC-3 CM
M
anti-Flt4
4 5 6
m.w.
170
116
PerVO4
PC-3 CM
M
B
anti-PTyr
1 2
m.w.
170
116
PerVO4
PC-3 CM
C
anti-PTyr
1 2 3 4 5
m.w.
6 7 8 9
170
116
PC-3 CM - + + - +
Heparin-Sepharose - - + - -
NIH 3T3/ Flt4
NIH 3T3
- + - +
NIH 3T3/ Flt4
NIH 3T3/ FGFR-4

Figure 5

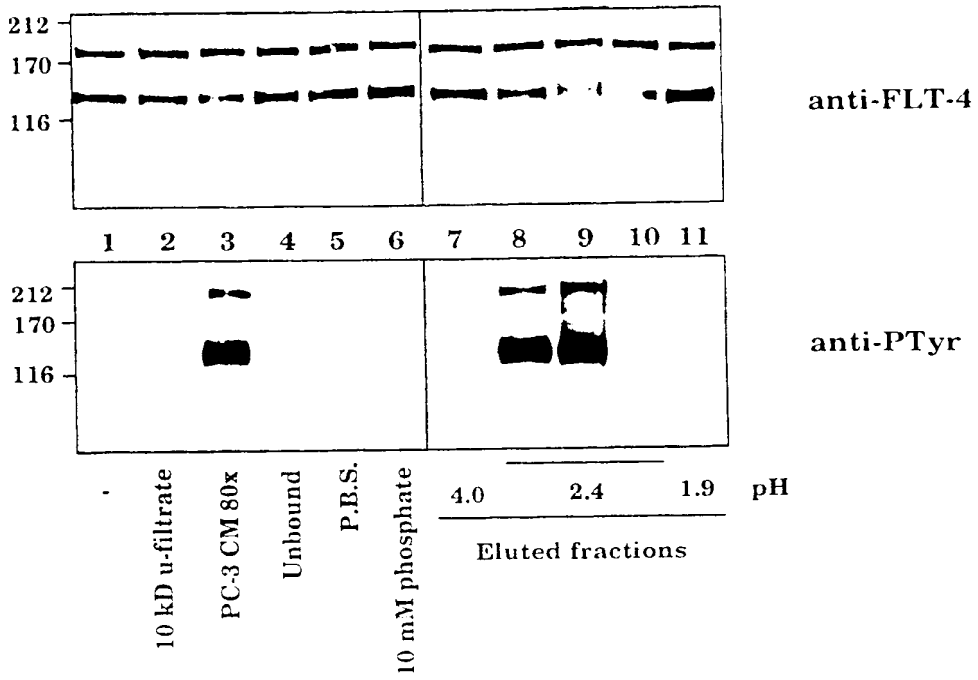

212
170
116
anti-FLT-4
1 2 3 4 5 6 7 8 9 10 11
212
170
116
anti-PTyr
-
10 kD u-filtrate
PC-3 CM 80x
Unbound
P.B.S.
10 mM phosphate
4.0
2.4
1.9
pH
Eluted fractions

Figure 6

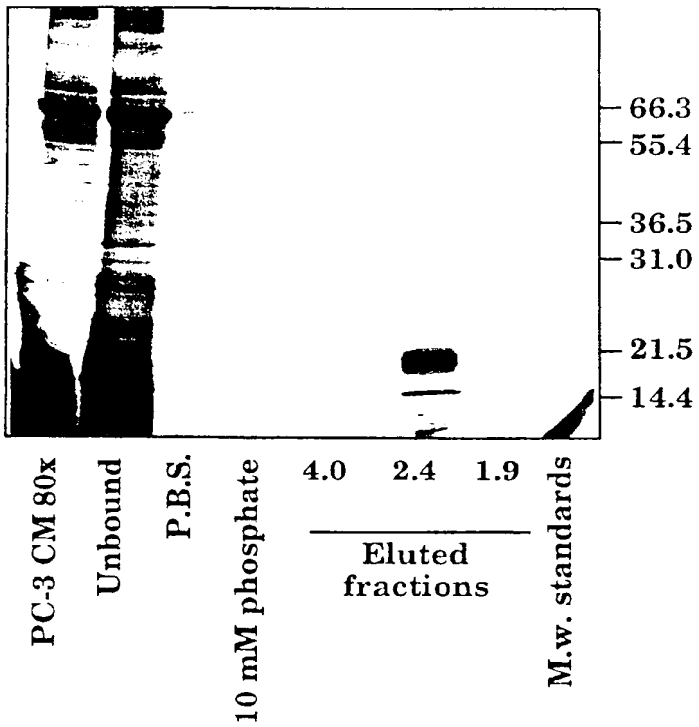

66.3
55.4
36.5
31.0
21.5
14.4
PC-3 CM 80x
Unbound
P.B.S.
10 mM phosphate
4.0
2.4
1.9
Eluted fractions
M.w. standards

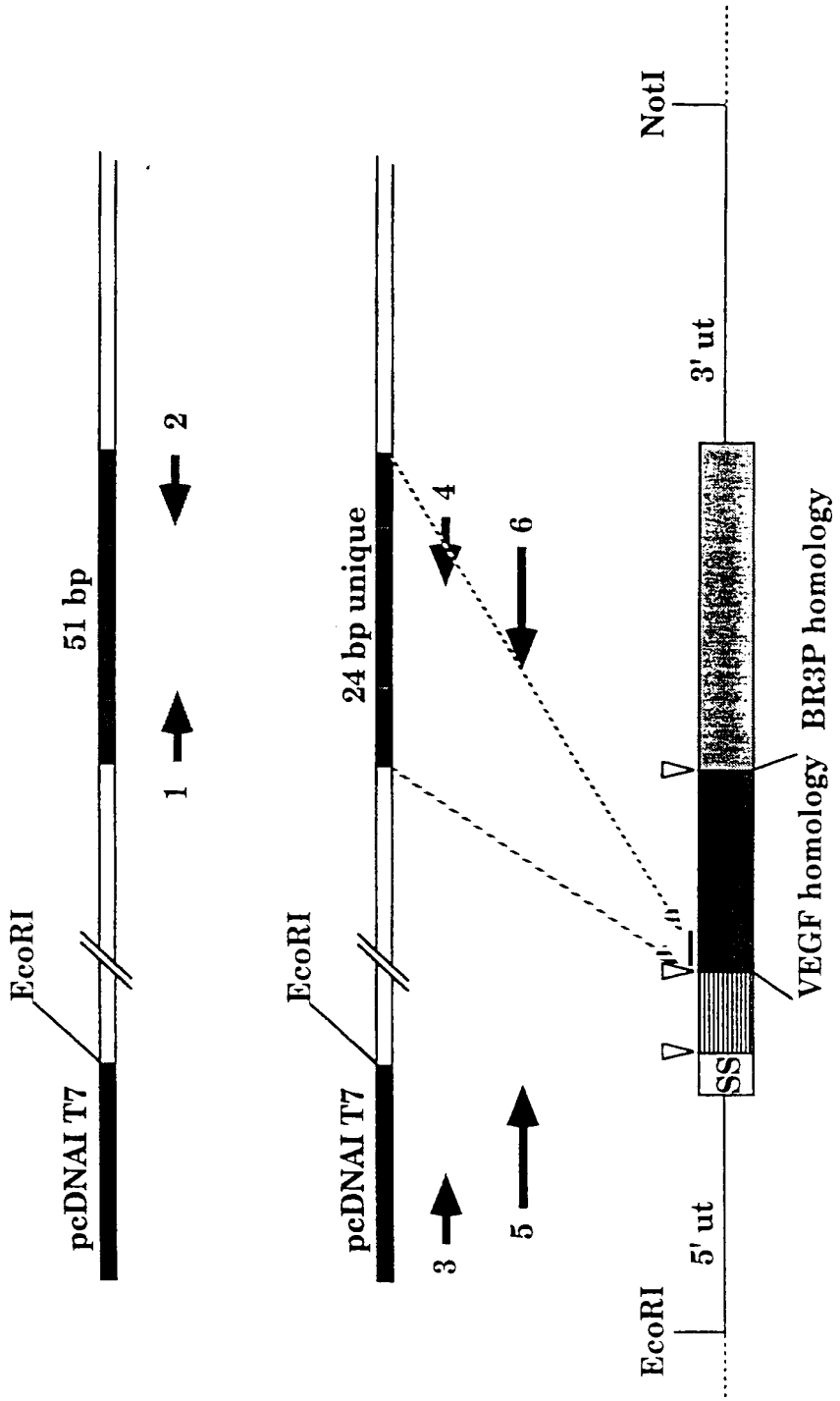
Figure 7

VEGF-C alignment

```
      1                                                  50
Hum   HMLLGFFSVA CSLLAAALLP GPREAPAAAA AFESGLDLSD AEPDAGEATA
Mou   MHLLCFLSLA CSLLAAALIP SPREAPATVA AFESGLGFSE AEPDGGEVKA
Qua   MHLLEMLSLG CCLAAGAVLL GPRQPPVA.A AYESGHGYYE EEPGAGEPKA

      51                                                 100
Hum   YASKDLEEQL RSVSSVDELM TVLYPEYWKM YKCQLRKGGW QHNREQANLN
Mou   FEGKDLEEQL RSVSSVDELM SVLYPDYWKM YKCQLRKGGW Q....QPTLN
Qua   HASKDLEEQL RSVSSVDELM TVLYPEYWKM FKCQLRKGGW QHNREHSSSD

      101                                                150
Hum   SRTEETIKFA AAHYNTEILK SIDNEWRKTQ CMPREVCIDV GKEFGVATNT
Mou   TRTGDSVKFA AAHYNTEILK SIDNEWRKTQ CMPREVCIDV GKEFGAATNT
Qua   TRSDDSLKFA AAHYNAEILK SIDTEWRKTQ GMPREVCVDL GKEFGATTNT

      151                                                200
Hum   FFKPPCVSVY RCGGCCNSEG LQCMNTSTSY LSKTLFEITV PLSQGPKPVT
Mou   FFKPPCVSVY RCGGCCNREG LQCMNTSTGY LSKTLFEITV PLSQGPKPVT
Qua   FFKPPCVSIY RCGGCCNSEG LQCMNISTNY ISKTLFEITV PLSHGPKPVT

      201                                                250
Hum   ISFANHTSCR CMSKLDVYRQ VHSIIRRSLP ATLPQCQAAN KTCPTNYMWN
Mou   ISFANHTSCR CMSKLDVYRQ VHSIIRRSLP ATLPQCQAAN KTCPTNYVWN
Qua   VSFANHTSCR CMSKLDVYRQ VHSIIRRSLP ATQTQCHVAN KTCPKNHVWN

      251                                                300
Hum   NHICRCLAQE DFMFSSDAGD DSTDGFHDIC GPNKELDEET CQCVCRAGLR
Mou   NYMCRCLAQQ DFIFYSNVED DSTNGFHDVC GPNKELDEDT CQCVCKGGLR
Qua   NQICRCLAQH DFGFSSHLGD SDTSEGFHIC GPNKELDEET CQCVCKGGVR

      301                                                350
Hum   PASCGPHKEL DRNSCQCVCK NKLFPSQCGA NREFDENTCQ CVCKRTCPRN
Mou   PSSCGPHKEL DRDSCQCVCK NKLFPNSCGA NREFDENTCQ CVCKRTCPRN
Qua   PISCGPHKEL DRASCQCMCK NKLLPSSCGP NKEFDEEKCQ CVCKKTCPKH

      351                                                400
Hum   QPLNPGKCAC ECTESPQKCL LKGKKFHHQT CSCYRRPCTN RQKACEPGFS
Mou   QPLNPGKCAC ECTENTQKCF LKGKKFHHQT CSCYRRPCAN RLKHCDPGLS
Qua   HPLNPAKCIC ECTESPNKCF LKGKRFHHQT CSCYRPPCTV RTKRCDAGFL

      401              420
Hum   YSEEVCRCVP SYWKRPQMS*
Mou   FSEEVCRCVP SYWKRPHLN.
Qua   LAEEVCRCVR TSWKRPLMN*
```

FIGURE 8

Figure 9


EcoRI
pcDNAI T7
51 bp
1
2
EcoRI
pcDNAI T7
24 bp unique
3
4
5
6
NotI
3' ut
BR3P homology
VEGF homology
SS
5' ut
EcoRI

```
            1                                                  50
PDGF-A      .......... .......... .......... .......... .MRTLACLLL
PDGF-B      .......... .......... .......... .......... MNRCWA.LFL
PlGF-1      .......... .......... .......... .......... ..........
VEGF165     .......... .......... .......... .......... ..........
VEGF-B167   .......... .......... .......... .......... ..........
VEGF-C      MHLLGFFSVA CSLLAAALLP GPREAPAAAA AFESGLDLSD AEPDAGEATA

            51                                                 100
PDGF-A      LGCGYLAHVL AEEAEIPREV IERLARSQIH SIRDLQRLLE IDSVGSEDSL
PDGF-B      SLCCYLRLVS AEGDPIPEEL YEMLSDHSIR SFDDLQRLLH GDP.GEEDGA
PlGF-1      .......... ......MPVM RLFPC..FLQ LLAGLAL... PAVPPQQW..
VEGF165     .......... .........M NFLLS..WVH WSLALLLYLH HAKWSQAA..
VEGF-B167   .......... .........M SPLLR..RLL LAALLQLAPA QAPVSQP...
VEGF-C      YASKDLEEQL RSVSSVDELM TVLYPEYWKM YKCQLRKGGW QHNREQANLN

            101                                                150
PDGF-A      DTSLRAHGVH ATKHVPEKRP LPIRRKRSI. .....EEAVP AVCKTRTVIY
PDGF-B      ELDLNMTRSH SGGELES... .LARGRRSLG SLTIAEPAMI AECKTRTEVF
PlGF-1      .....ALSAG NGSSEVEVVP FQE.VWGR.. .......... SYCRALERLV
VEGF165     .....PMAEG GGQNHHEVVK FMD.VYQR.. .......... SYCHPIETLV
VEGF-B167   .........D APGHQRKVVS WID.VYTR.. .......... ATCQPREVVV
VEGF-C      SRTEETIKFA AAHYNTEILK SIDNEWRK.. .......... TQCMPREVCI

            151                                                200
PDGF-A      EIPRSQVDPT SANFLIWPPC VEVKRCTGCC NTSSVKCQPS RVHHRSVKVA
PDGF-B      EISRRLIDRT NANFLVWPPC VEVQRCSGCC NNRNVQCRPT QVQLRPVQVR
PlGF-1      DVVSEYPSEV ..EHMFSPSC VSLLRCTGCC GDENLHCVPV ETANVTMQLL
VEGF165     DIFQEYPDEI ..EYIFKPSC VPLMRCGGCC NDEGLECVPT EESNITMQIM
VEGF-B167   PLTVELMGTV ..AKQLVPSC VTVQRCGGCC PDDGLECVPT GQHQVRMQIL
VEGF-C      DVGKEFGVAT ..NTFFKPPC VSVYRCGGCC NSEGLQCMNT STSYLSKTLF
```

FIG. 10 (1 of 3)

```
      201                                                        250
PDGF-A    KVEYVRKKPK LKEVQVRLEE HLECACAT.. .......... TSLNPDYREE
PDGF-B    KIEIVRKKPI FKKATVTLED HLACKCETVA AARPVTRSPG GSQEQRAKTP
P1GF-1    KIRSG..DRP .SYVELTFSQ HVRCECRPLR EK........ ..........
VEGF165   RIKPH..QGQ .HIGEMSFLQ HNKCECRPKK DR........ ..........
VEGF-B167 MIRYP..SSQ ..LGEMSLEE HSQCECRPKK KD........ ..........
VEGF-C    EITVPLSQGP .KPVTISFAN HTSCRCMSKL DVYRQVHSII RRSLPATLPQ

          251                                                  300
PDGF-A    DTDVR..... .......... .......... .......... ..........
PDGF-B    QTRVTIRTVR VRRPPKGKHR KFKHTHDKTA LKETLGA... ..........
P1GF-1    .......... .......... .......... .MKPERCGDA VPRR......
VEGF165   .......... .......... .......... .ARQENPCGP CSERRKHLFV
VEGF-B167 .......... .......... .........S AVKPDSPRPL CPRCTQHHQR
VEGF-C    CQAANKTCPT NYMWNNHICR CLAQEDFMFS SDAGDDSTDG FHDICGPNKE

          301                                                  350
PDGF-A    .......... .......... .......... .......... ..........
PDGF-B    .......... .......... .......... .......... ..........
P1GF-1    .......... .......... .......... .......... ..........
VEGF165   QDPQTCKCSC KNTDS.RCKA RQLELNERTC RCDKPRR... ..........
VEGF-B167 PDPRTCRCRC RRRSFLRCQG RGLELNPDTC RCRKLRR... ..........
VEGF-C    LDEETCQCVC RAGLRPASCG PHKELDRNSC QCVCKNKLFP SQCGANREFD
```

FIG. 10 (2 of 3)

```
                351                                                400
PDGF-A  ..........  ..........  ..........  ..........  ..........  ..........
PDGF-B  ..........  ..........  ..........  ..........  ..........  ..........
P1GF-1  ..........  ..........  ..........  ..........  ..........  ..........
VEGF165 ..........  ..........  ..........  ..........  ..........  ..........
VEGF-B167 ..........  ..........  ..........  ..........  ..........  ..........
VEGF-C  ENTCQCVCKR TCPRNQPLNP GKCACECTES PQKCLLKGKK FHHQTCSCYR

                401                                                434
PDGF-A  ..........  ..........  ..........  ..........  ..........  ....
PDGF-B  ..........  ..........  ..........  ..........  ..........  ....
P1GF-1  ..........  ..........  ..........  ..........  ..........  ....
VEGF165 ..........  ..........  ..........  ..........  ..........  ....
VEGF-B167 ..........  ..........  ..........  ..........  ..........  ....
VEGF-C  RPCTNRQKAC EPGFSYSEEV CRCVPSYWKR PQMS
```

FIG. 10 (3 of 3)

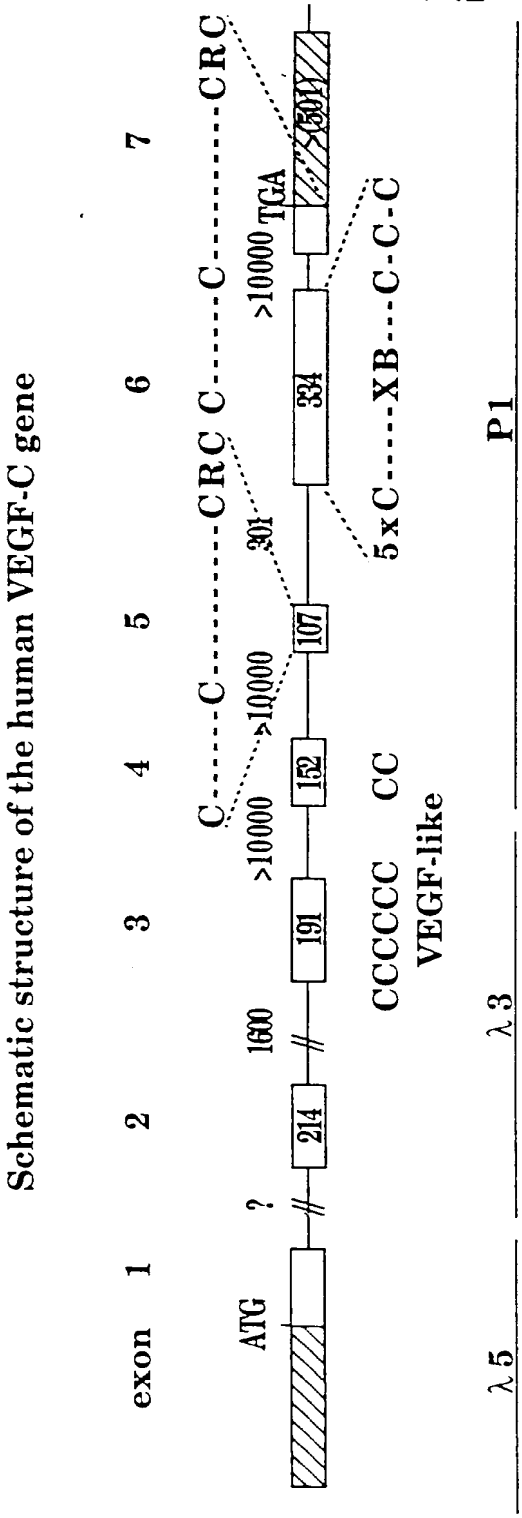

Schematic structure of the human VEGF-C gene
exon 1 2 3 4 5 6 7
ATG
?
1600
214
191
>10000
C
>10000
152
>10000
C
301
107
CRC
C
>10000
TGA
334
CRC
5xC
XB
C-C-C
CCCCCC
CC
VEGF-like
λ5
λ3
P1

FIGURE 11

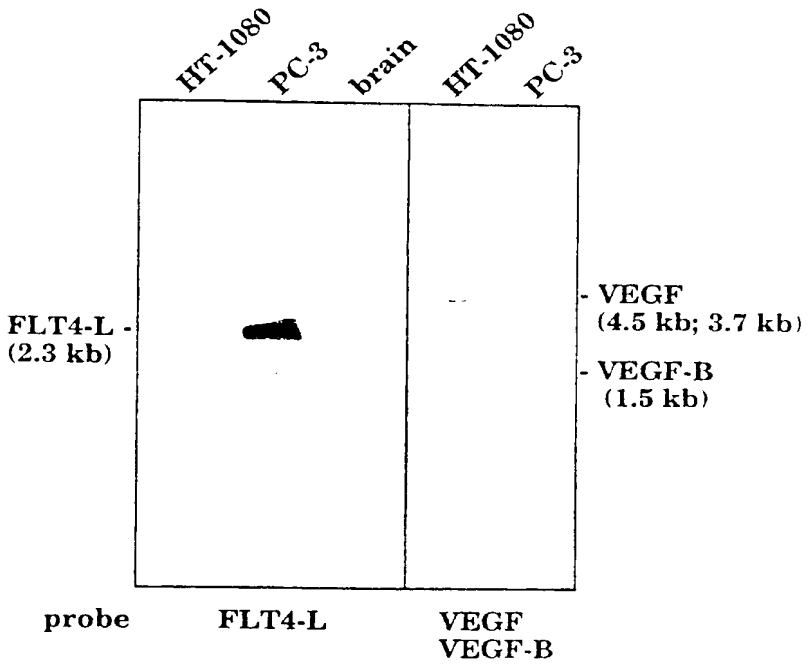

HT-1080
PC-3
brain
HT-1080
PC-3
FLT4-L -
(2.3 kb)
- VEGF
(4.5 kb; 3.7 kb)
- VEGF-B
(1.5 kb)
probe
FLT4-L
VEGF
VEGF-B

Figure 12

A

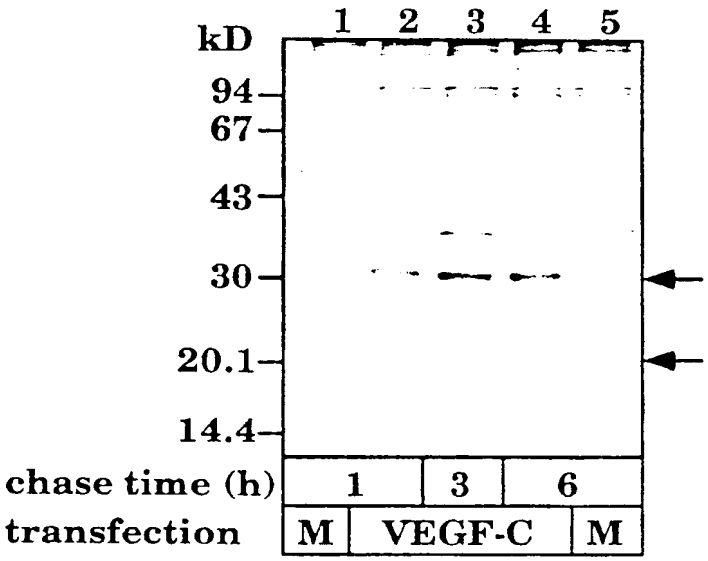

1 2 3 4 5
kD
94
67
43
30
20.1
14.4
chase time (h)
1
3
6
transfection
M
VEGF-C
M

Figure 13A

B

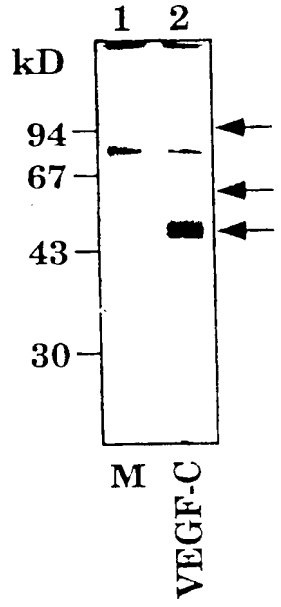


Figure 13B

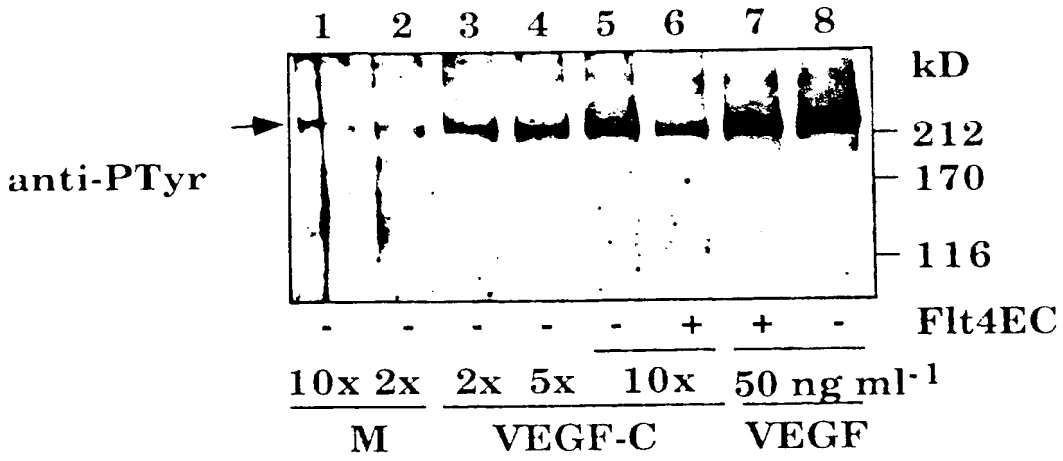

1 2 3 4 5 6 7 8
kD
212
170
116
anti-PTyr
- - - - - + + -
Flt4EC
10x 2x 2x 5x 10x 50 ng ml-1
M VEGF-C VEGF

Figure 14A

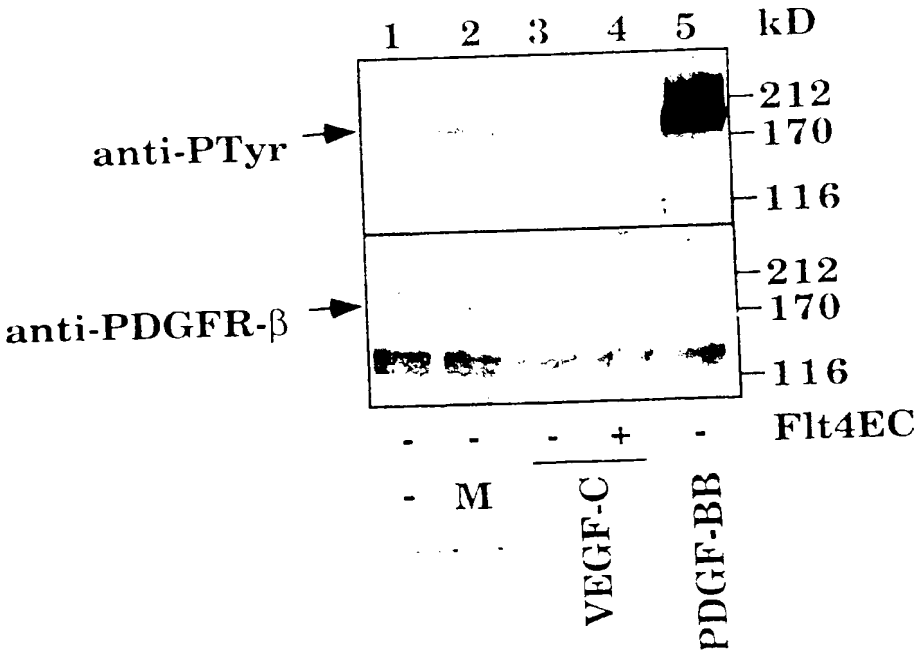

1 2 3 4 5 kD
anti-PTyr
212
170
116
anti-PDGFR-β
212
170
116
Flt4EC
- - - + -
- M
VEGF-C
PDGF-BB

Figure 14B

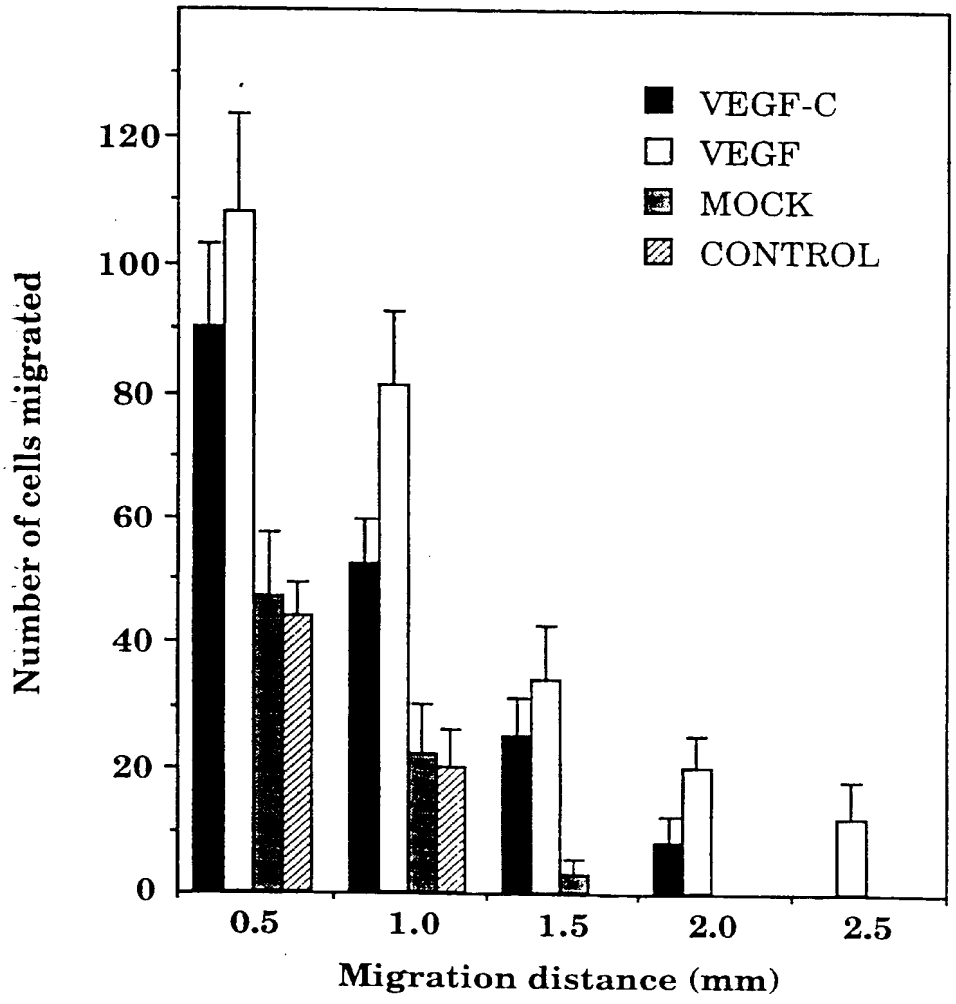

VEGF-C
VEGF
MOCK
CONTROL
Number of cells migrated
0
20
40
60
80
100
120
0.5
1.0
1.5
2.0
2.5
Migration distance (mm)

Figure 15

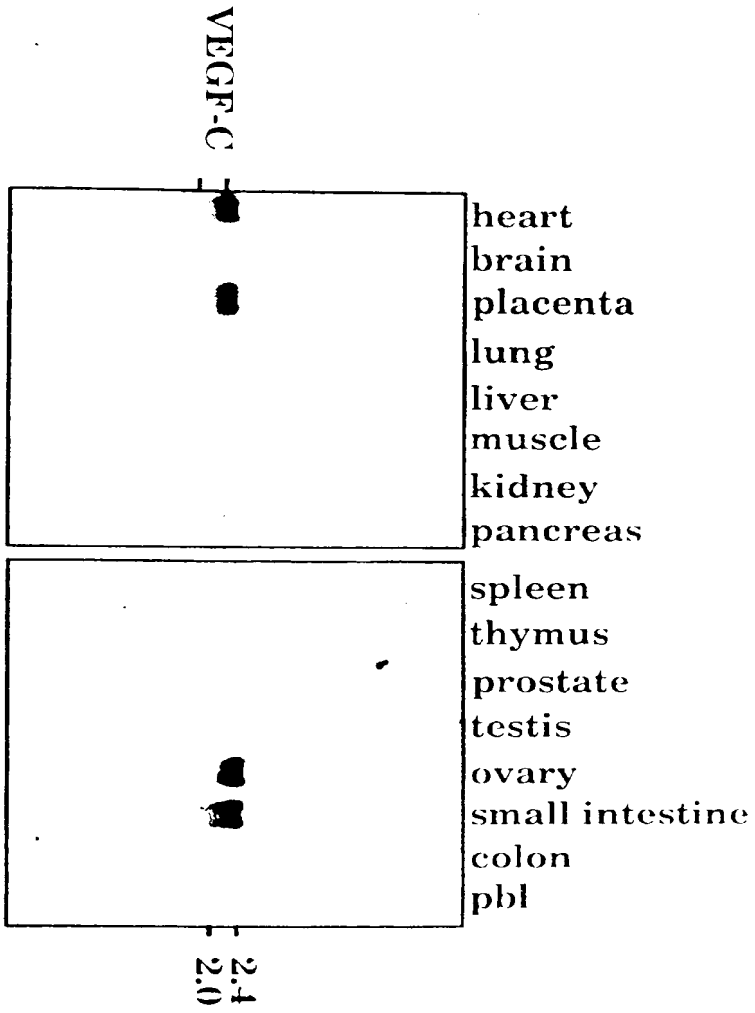

VEGF-C
heart
brain
placenta
lung
liver
muscle
kidney
pancreas
spleen
thymus
prostate
testis
ovary
small intestine
colon
pbl
2.4
2.0

Figure 16A

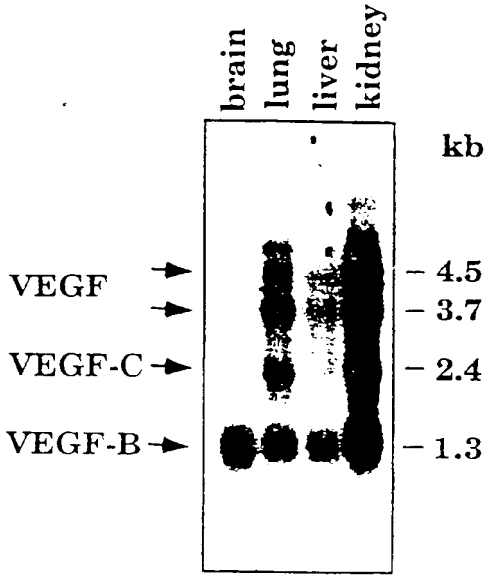


FIG. 16B

HUMAN

Exon length **Donor site** **Intron length** **Acceptor site**

```
.....G...E...A...T(49).....A...Y...A...S.
E1.....GGC.GAG.GCC.ACG.gtaggtctgcgt...>10.kb..TTTCTTTGACAG.GCT.TAT.GCA.AGC
.....E...I...L...K(116).....S...I...D...N.
E2.214.bp..GAG.ATC.TTG.AAA.Agtaagtatggg.....4.kb....atgacttgacagGT.ATT.GAT.AAT
.....L...S...K...T(180).....L...F...E...I.
E3.191.bp..CTC.AGC.AAG.ACG.gtgggtattgt.....9.kb.cccttctttgtag.TTA.TTT.GAA.ATT
.....T...L...P...Q(231).....C...Q...A...A.
E4.152.bp..ACA.CTA.CCA.CAgtgagtatgaattaaa.>10.kb..ttcttccaaagG.TGT.CAG.GCA.GCG
.....A...G...D...(266).....D...S...T...D.
E5.107.bp..GCT.GGA.GAT.Ggtagcagaatg.....301.bp....ctatttgtctagAC.TCA.ACA.GAT
.....Q...T...C...S(378).....C...Y...R...R.
E6.334.bp..CAA.ACA.TGC.AGgtaagagatcc.....>10.kb..tgttctcctagC.TGT.TAC.AGA.CGG
.....Q...M...S(415)Stop.....
E7.(501).bp..CAA.ATG.AGC.TAA.GTATGTACTGTT...ATTGTATTAT
```

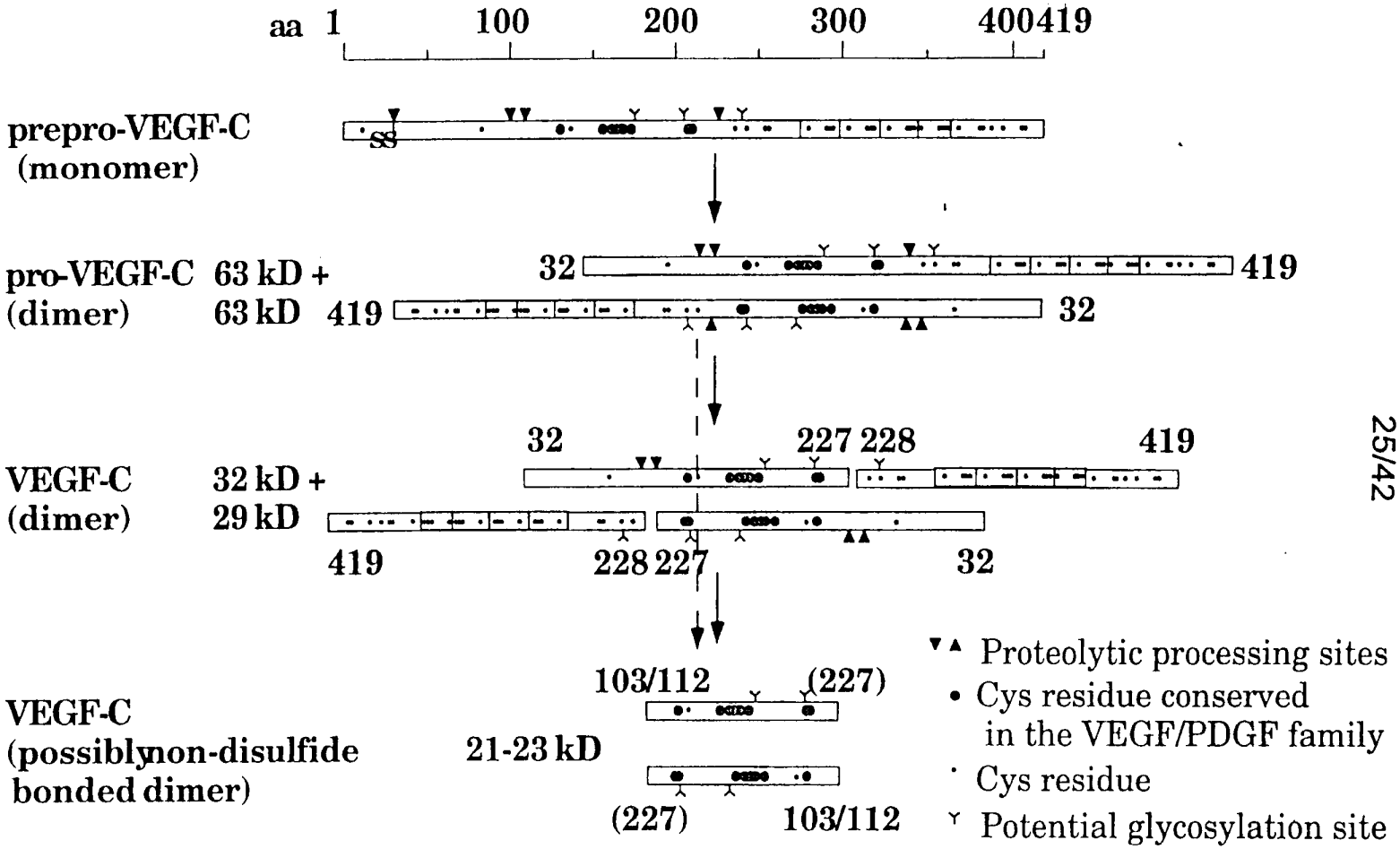
Figure 17 (1 of 2)

MOUSE

Exon length	Donor site	Intron length	Acceptor site
..........G...E...V...K(49)			.A...F...E...G.
E1.....GGC.GAG.GTC.AAG.	gtaggtgcaagg.	>10.kb.	attgtctttgacag.GCT.TTT.TGA.AGG
..........E...I...L...K(116)			.S...I...D...N.
E2.201.bp..GAG.ATC.CTG.AAA.	Agtaagtag.....	4.kb....	tgtgactcgacagGT.ATT.GAT.AAT
..........L...S...K...T(180)			.L...F...E...I.
E3.191.bp..CTC.AGC.AAG.ACG.	gtaggtat.....	9.kb..	ttgtcccttgtag.TTG.TTT.GAA.ATT
..........T...L...P...Q(231)			.C....Q...A...A.
E4.152.bp..ACA.TTA.CCA.CA	gtgagtatg.....	10.kb.	gtctccccaaaagG.TGT.CAG.GCA.GCT
..........N...V...E...D(266)			.D....S...T...N.
E5.107.bp..AAT.GTT.GAA.GAT.	Ggtaagtaaaa...	350.bp.....	tctagAC.TCA.ACC.AAT
..........Q...T...C...S(378)			.C...Y...R...R.
E6.334.bp..CAA.ACA.TGC.AG	gtaaggagtgt.....	6.kb..	ttttcccctagT.TGT.TAC.AGA.AGA
..........H...L...N(415)Stop			.....polyA.....
E7.506.bp..CAT.CTG.AAC.TAA.GATCATACC...ATTGTATTATAAgctgtgaag			

Figure 17 (2 of 2)

Figure 18


aa 1
100
200
300
400419
prepro-VEGF-C
(monomer)
ss
pro-VEGF-C 63 kD +
(dimer) 63 kD 419
32
419
32
VEGF-C 32 kD +
(dimer) 29 kD
32
227 228
419
419
228 227
32
VEGF-C
(possiblynon-disulfide
bonded dimer)
21-23 kD
103/112
(227)
(227)
103/112
Proteolytic processing sites
Cys residue conserved
in the VEGF/PDGF family
Cys residue
Potential glycosylation site

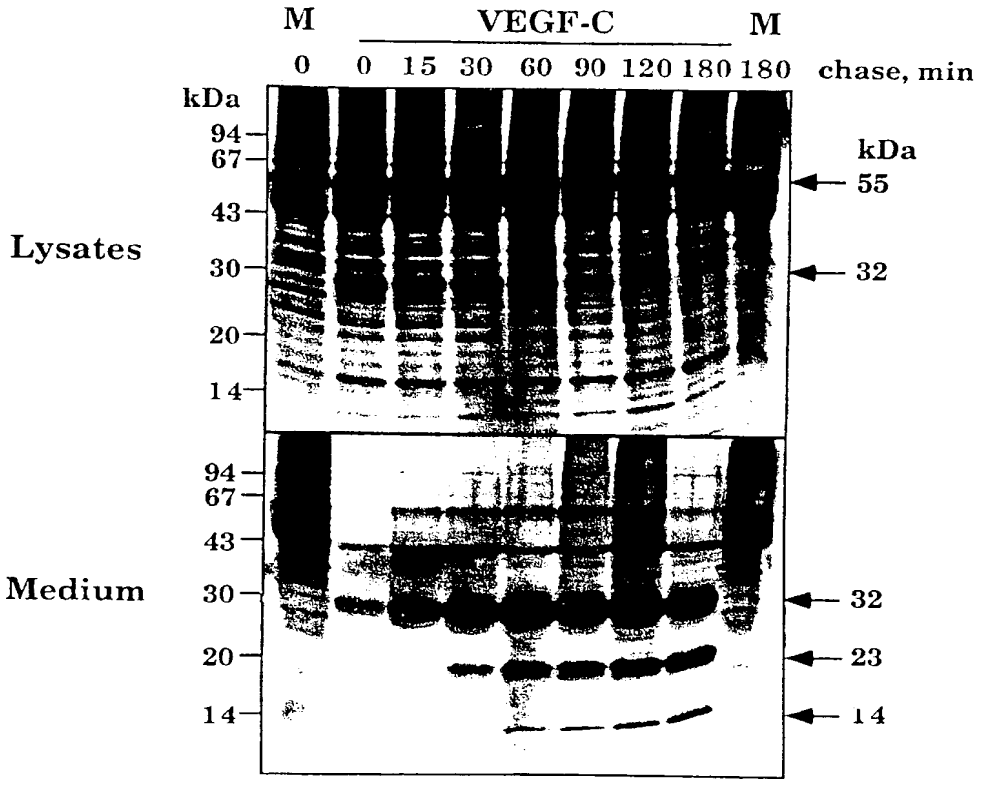

M
VEGF-C
M
0
0 15 30 60 90 120 180 180 chase, min
kDa
94
67
43
30
20
14
Lysates
Medium
kDa
55
32
32
23
14

Figure 19

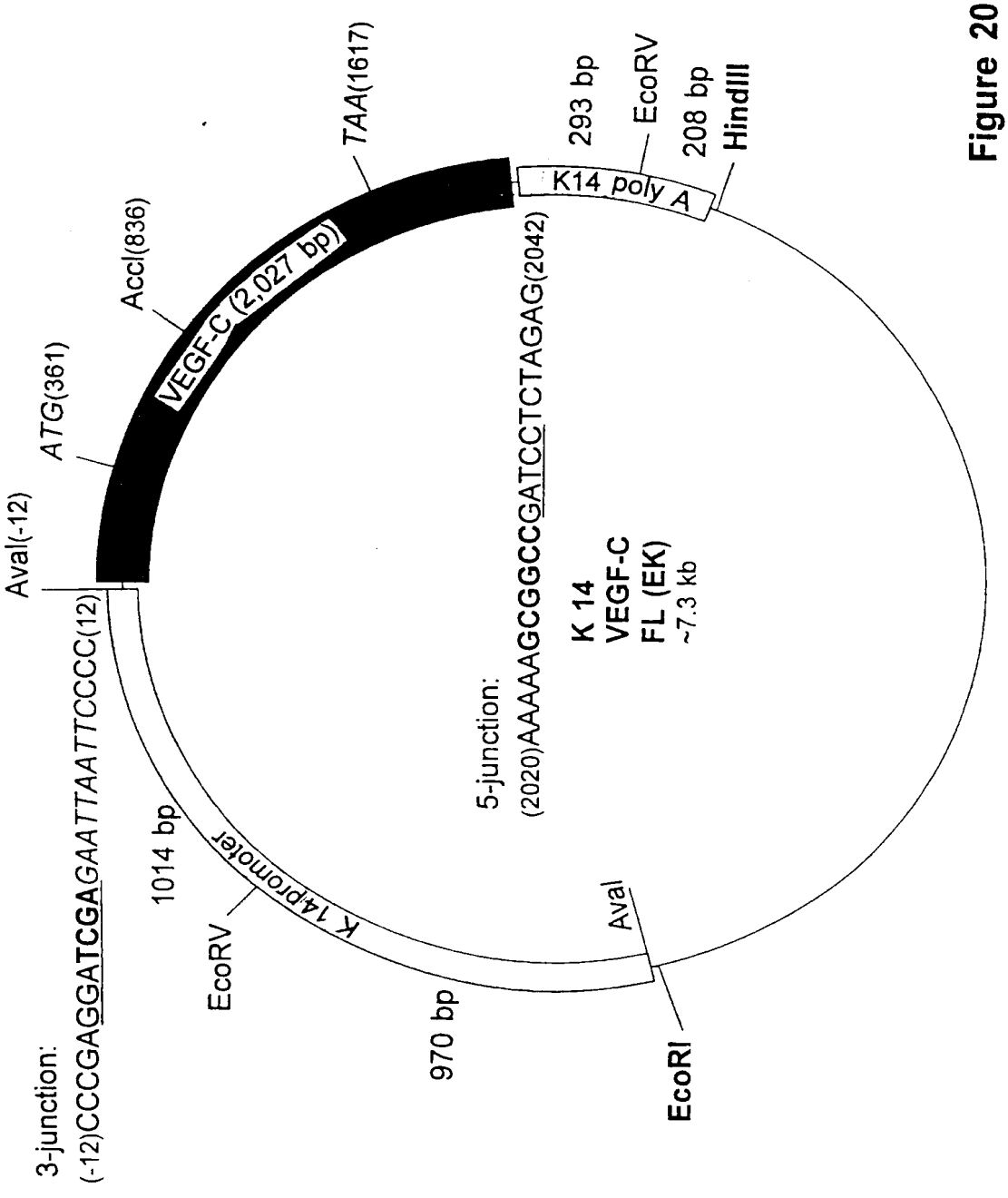
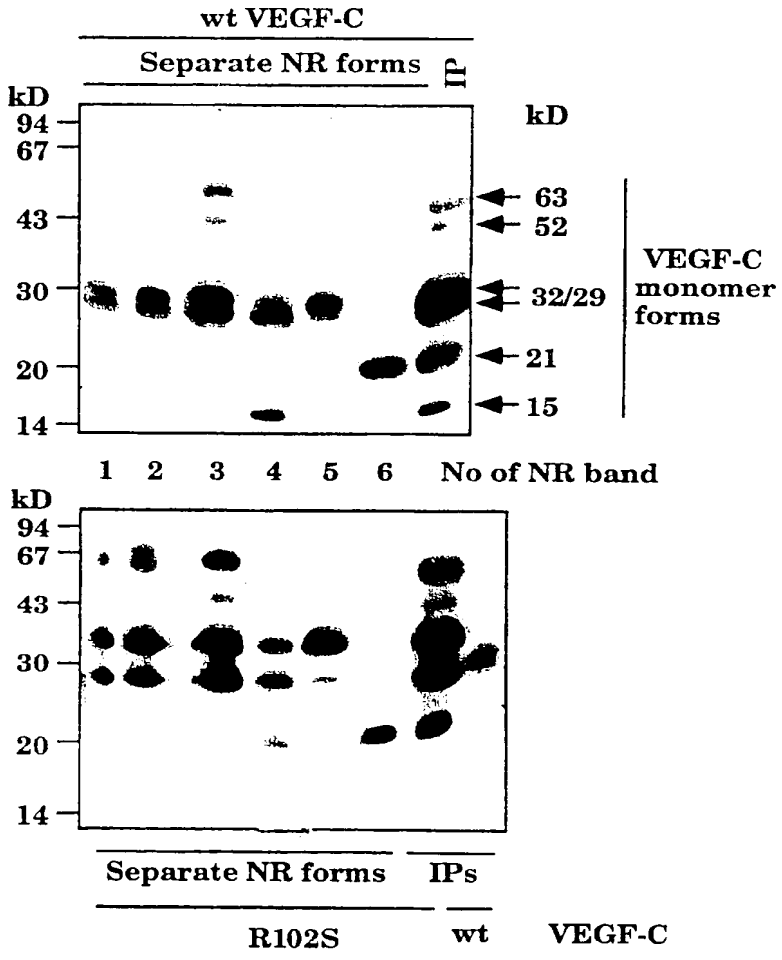

TAA(1617)
293 bp
EcoRV
208 bp
HindIII
K14 poly A
AccI(836)
VEGF-C (2,027 bp)
ATG(361)
AvaI(-12)
5-junction:
(2020)AAAAGCGGCCGATCCTCTAGAG(2042)
K 14
VEGF-C
FL (EK)
~7.3 kb
3-junction:
(-12)CCCGAGGATCGAATTAATTCCCC(12)
1014 bp
K 14promoter
EcoRV
970 bp
AvaI
EcoRI

Figure 20

Reducing

Figure 21A



Non-reducing

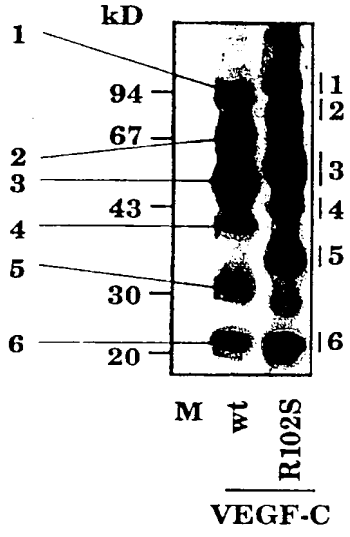


Figure 21B

Figure 21C

Media

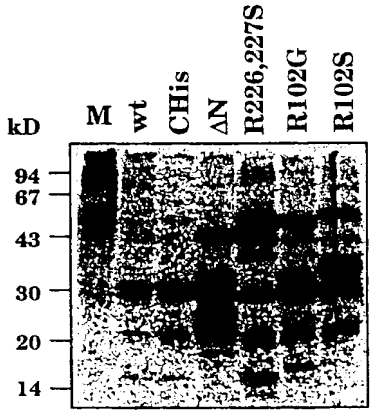


FIGURE 22A

Lysates

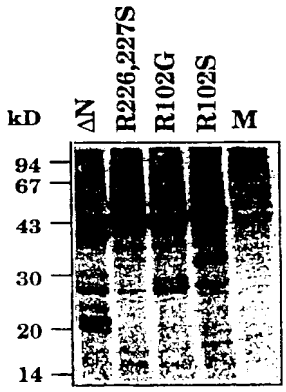


FIGURE 22B

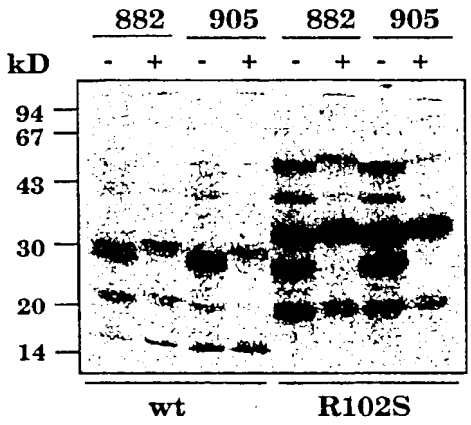


FIGURE 23A

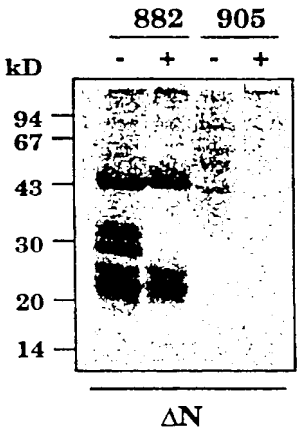


FIGURE 23B

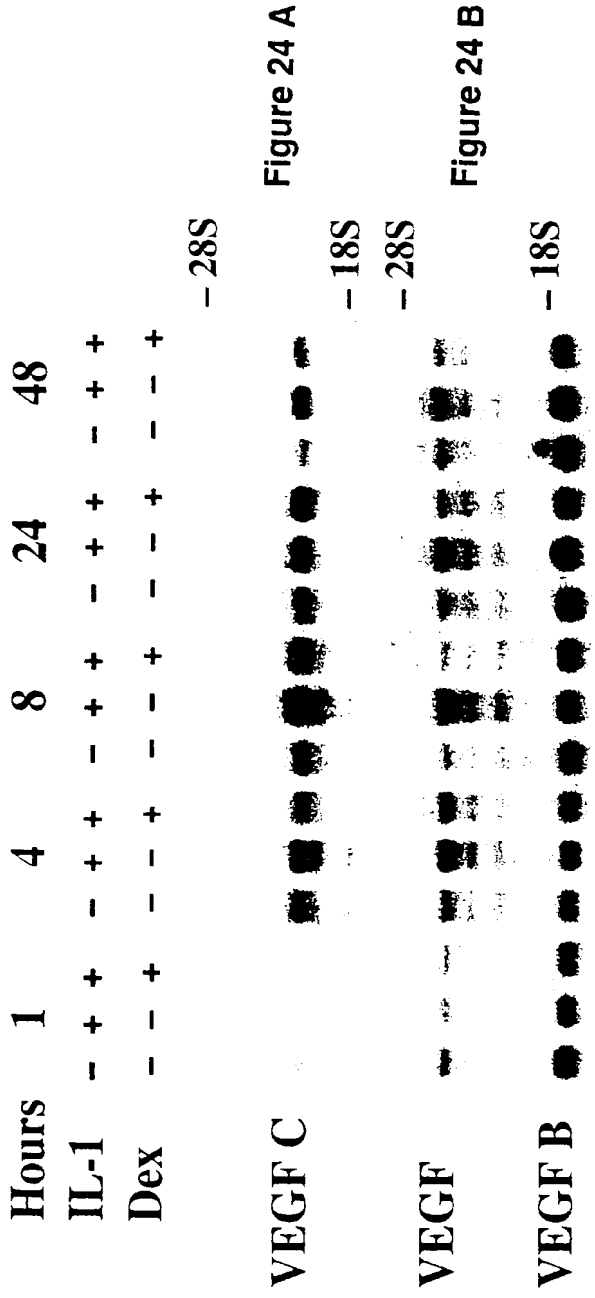


Figure 24 A

Figure 24 B

Figure 25

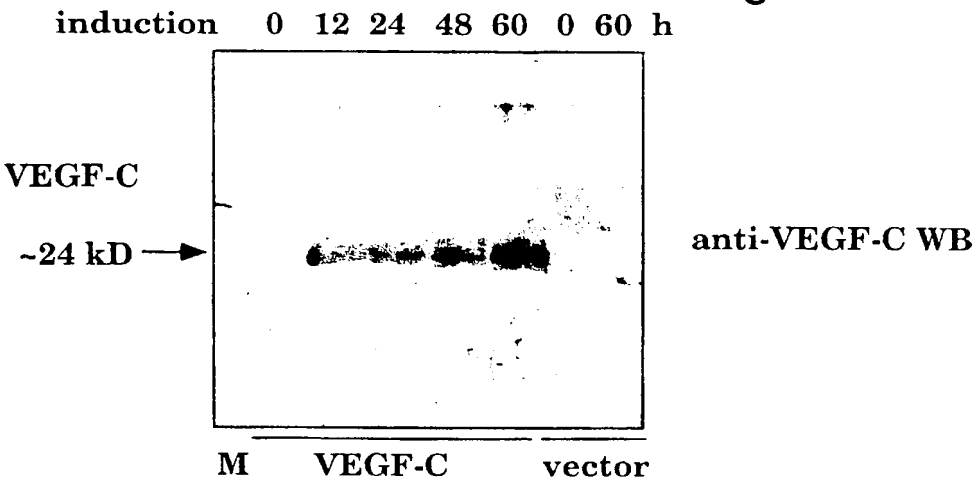
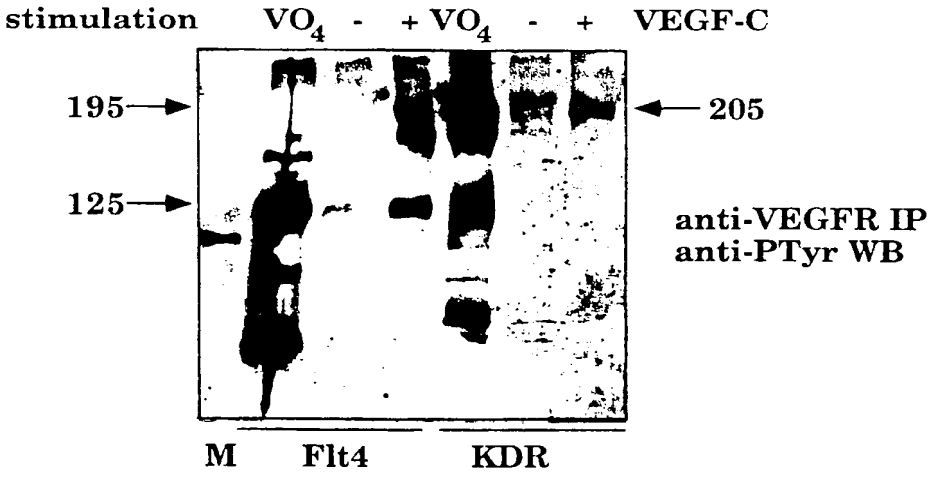


Figure 26



Media

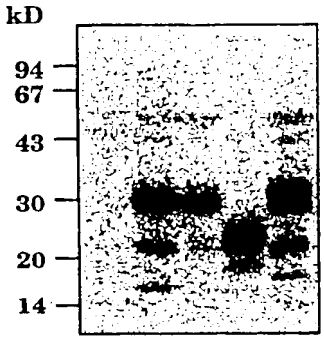


FIGURE 27A

Lysates

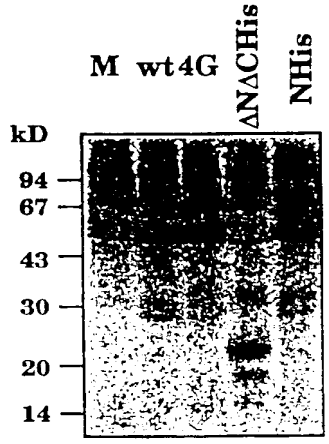


FIGURE 27B

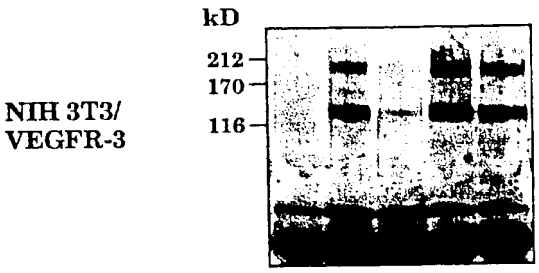


FIGURE 28A

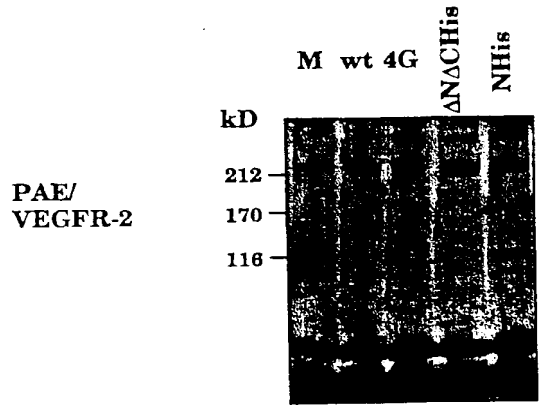


FIGURE 28B

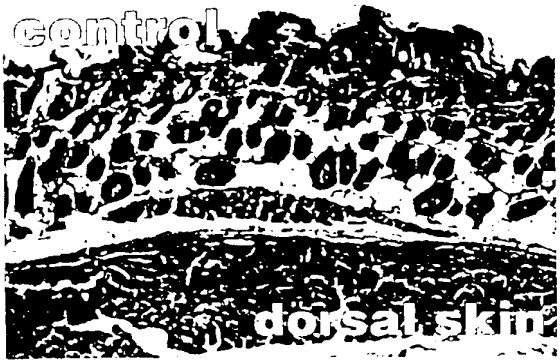


Figure 29A

Figure 29B

Figure 29C

Figure 29D

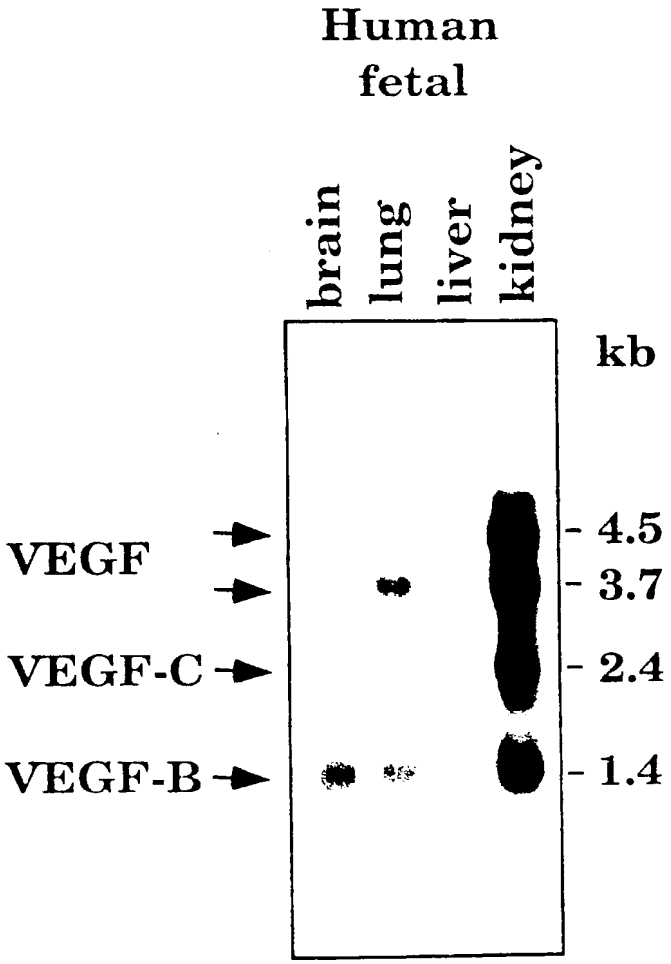

Human
fetal
brain
lung
liver
kidney
kb
VEGF
VEGF-C
VEGF-B
4.5
3.7
2.4
1.4

Figure 30

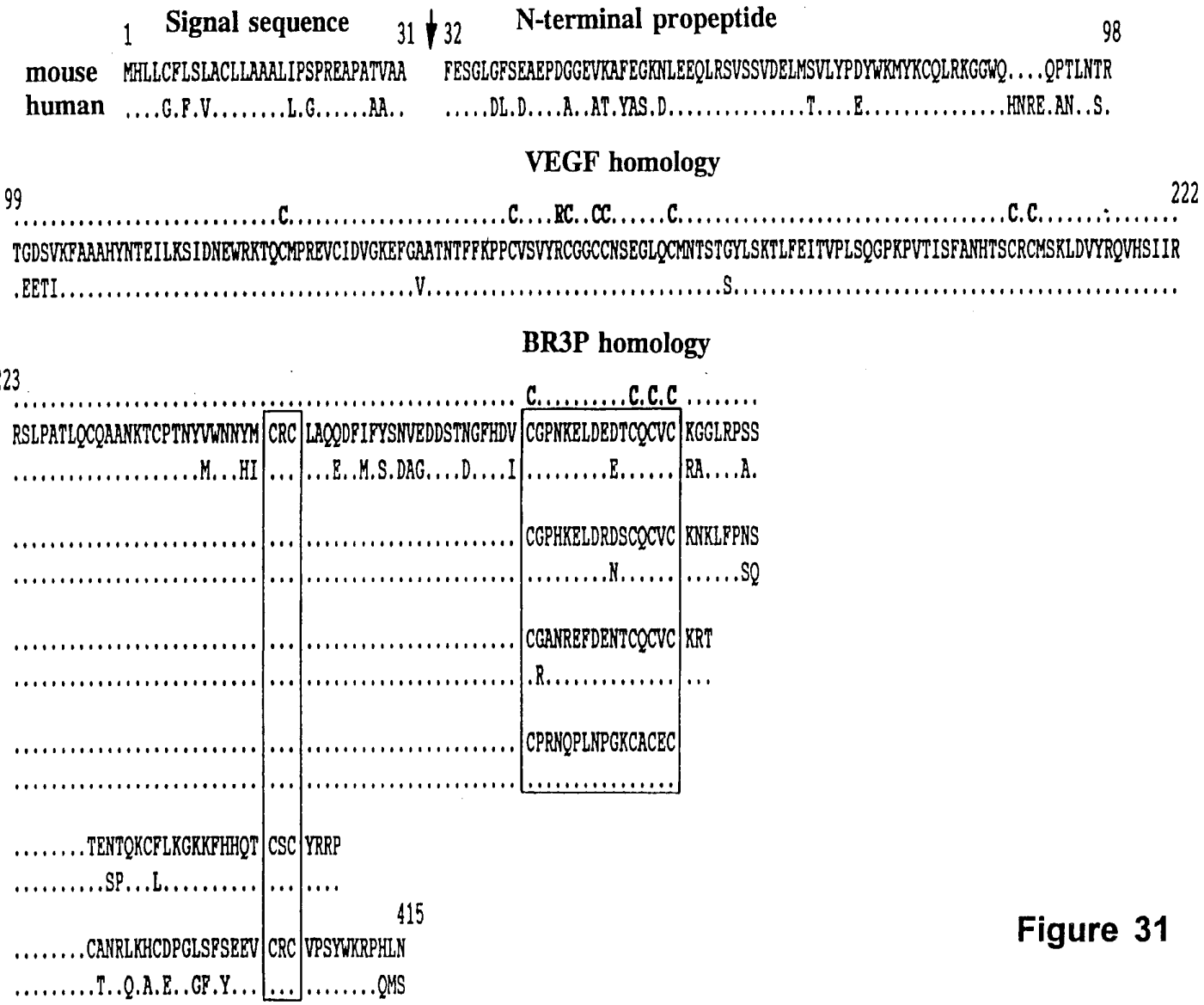


Figure 31

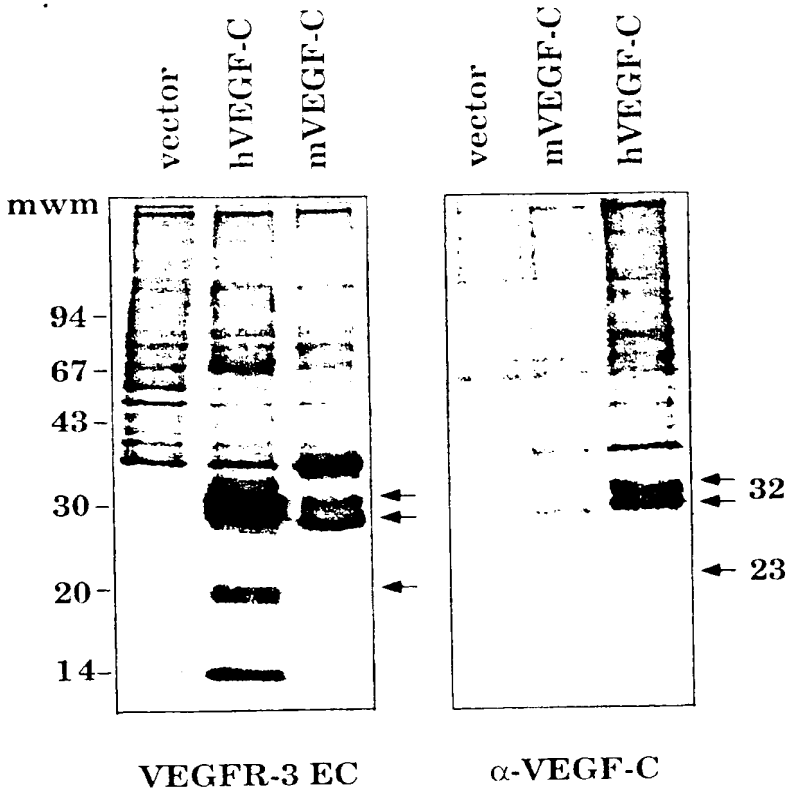

vector
hVEGF-C
mVEGF-C
vector
mVEGF-C
hVEGF-C
mwm
94
67
43
30
20
14
32
23
VEGFR-3 EC
α-VEGF-C

Figure 32

Figure 33

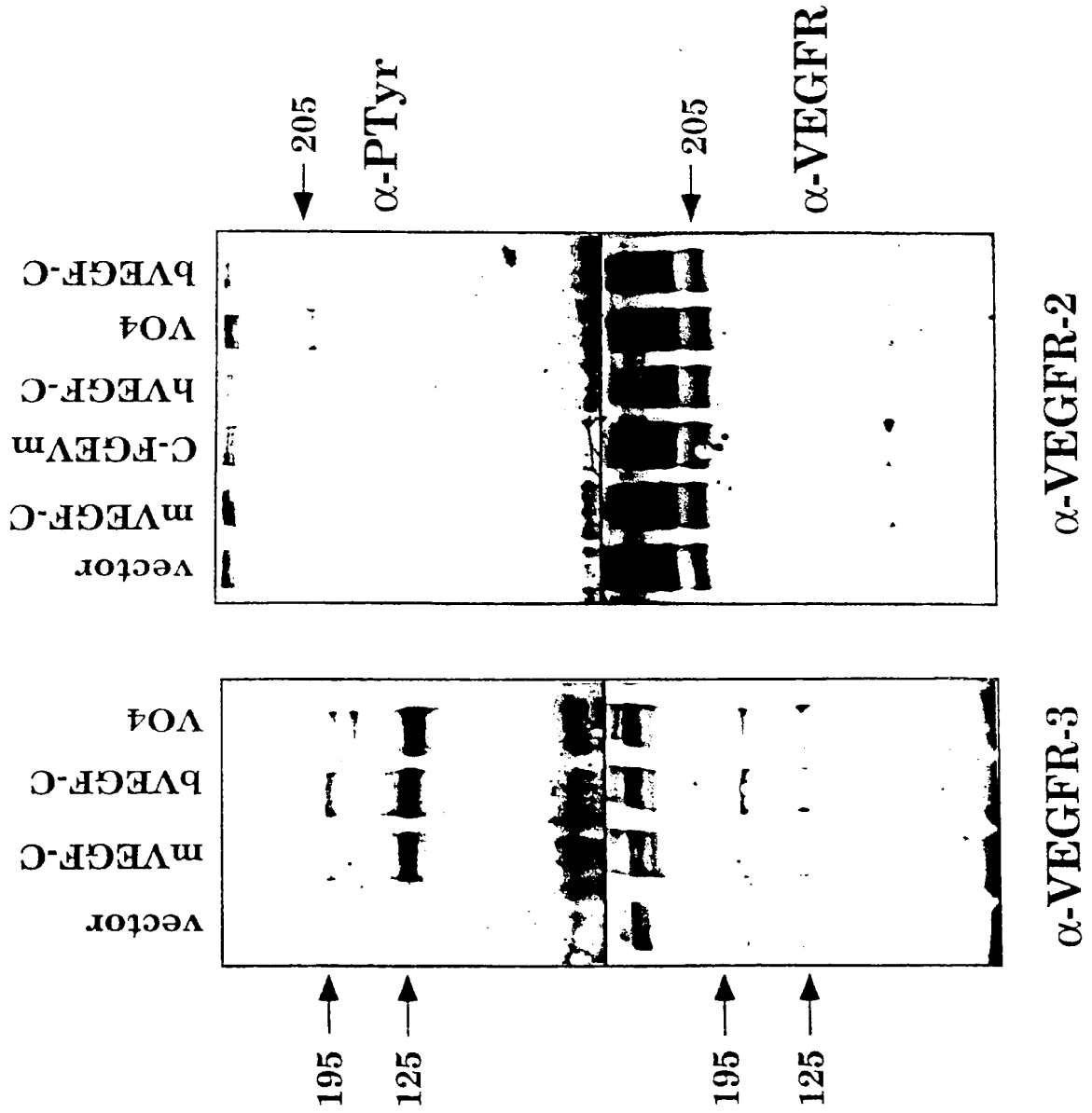

205
α-PTyr
205
α-VEGFR
vector
mVEGF-C
C-FGEVm
hVEGF-C
VO4
hVEGF-C
α-VEGFR-2
vector
mVEGF-C
hVEGF-C
VO4
α-VEGFR-3
195
125
195
125

Figure 34A

Figure 34B

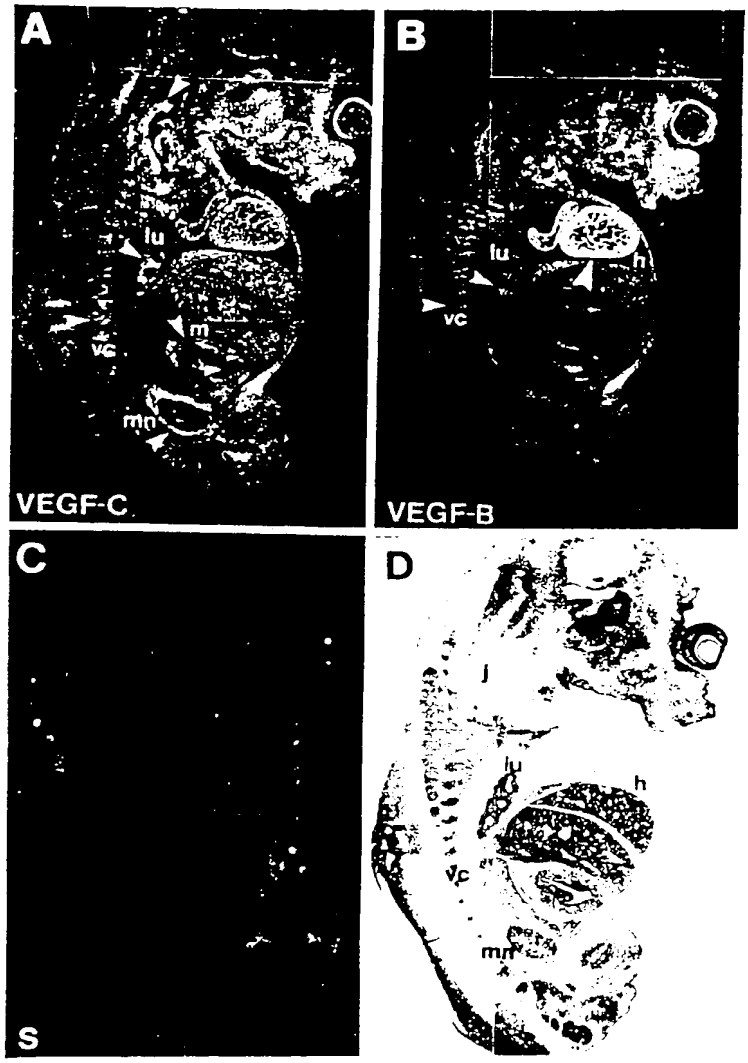


Figure 34C

Figure 34D

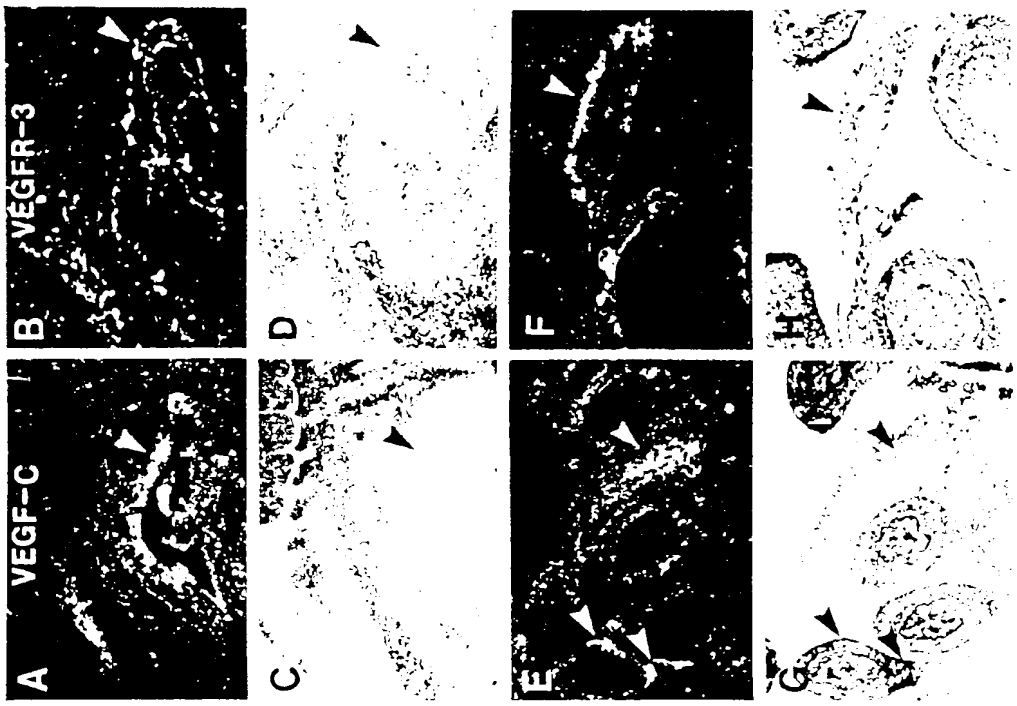


Figure 35A Figure 35B

Figure 35C Figure 35D

Figure 35E Figure 35F

Figure 35G Figure 35H

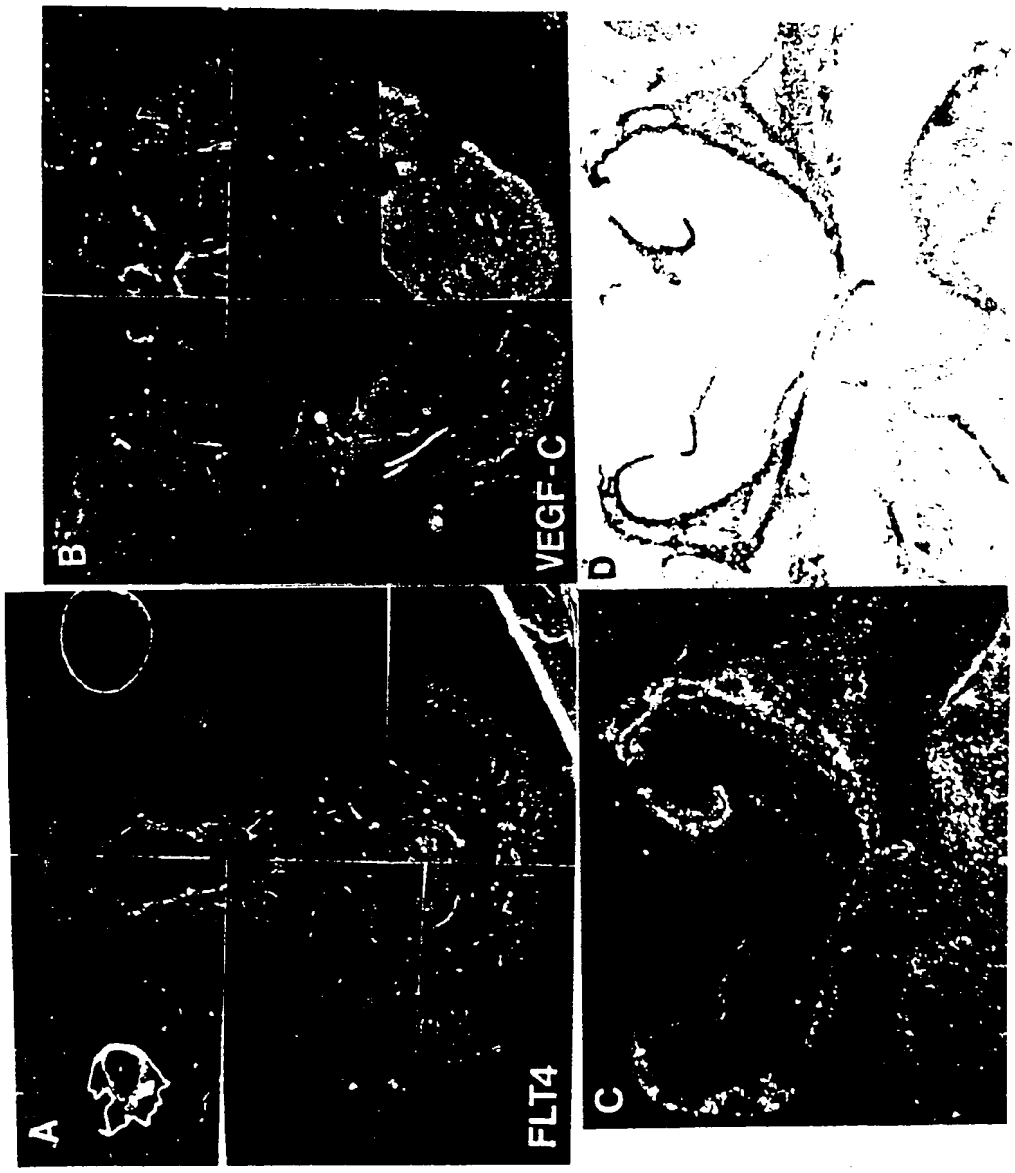


Figure 36A

Figure 36B

Figure 36C

Figure 36D